Poultry, Fisheries & Wildlife Sciences
ISSN: 2375-446X

Daud Om et al., Poult Fish Wildl Sci 2016, 4:2
DOI: 10.4172/2375-446X.1000163

Research Article Open Access

Use of Vitellogenin as Biomarker Indicator in Sex Identification of Giant Grouper (*Epinephelus lanceolatus*)

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Abstract

Vitellogenin, the egg yolk precursor protein, has studied as biomarker for sex identification of giant grouper (*Epinephelus lanceolatus*). Vitellogenin was synthesized successfully in liver of male giant grouper by implanted with 2 mg/kg of Estradiol-17-ß (E2). The levels of vitellogenin (vtg) in blood of E2-injected fish were comparable with those found in the blood of naturally male and female by observed with SDS-PAGE.

With antigen from purified plasma E2-treated was produced polyclonal antibody of giant grouper after immunization process with rabbit for 72 days. Analyst by ELISA has demonstrated polyclonal antibody was developed within 2 weeks after primary immunization and greatest differences in absorbance at 1:8,000 dilutions. Validation by western blot, has verified vitellogenin as positive control (in female natural) and negative control (in male natural). It's showed that the antigens vtg of giant grouper polyclonal antibody recognized the purified giant grouper vtg in natural female and treated estradiol male.

The information on Vtg through this experiment could provide the basis of an immunological assay for sex identification and maturational status of the Giant grouper. Consequently, the aim of the present study was to establish a simple, efficient and non-lethal technique by using Vtg as biomarker indicator for rapid sex identification without killing or seriously injuring the sampled individuals.

Keywords: Vitellogenin; Giant grouper; Polyclonal antibody; ELISA; Western Blot

Introduction

In general, identification of sex and sexual maturity of the Giant grouper is difficult, because both males and females are found simultaneously and there is no definite size or age at which the sex reversal begins. In some cases, biopsies or endoscopies technique allowed sex identification [1]; however, most of the procedures were depended on a reliable method. This method is limited because histological technique is labor-intensive, expensive and slow to generate sufficient usable data. An easily identifiable marker for the onset of maturation in female fish has to be discovered.

Currently, there are several techniques used for sex identifications in fish. One example, the amplified fragment length polymorphism (AFLP) technique, generates large numbers of molecular markers and provides a rapid method for scanning the genomes of different individuals for sequence variation. The AFLP technique can be used to quantify genetic variation, and assist in marker-assisted breeding programs. In some cases, sex identification is an integral part of the characterization of forensic samples containing genomic DNA. PCR technique (Nagahama, 2005) offers an efficient and sensitive method for sex identification by amplifying gender-specific sequences, which results in different size PCR product depending on the gender of the donor. Genetic sex identification in Japanese Medaka is based on the presence or absence of the medaka male-sex determining gene, DMY, which is located on the Y chromosome.

Genetic identification of sex has been used to distinguish gonad identification. Analysis of chromosomes (X and Y) by using PCR could be an efficient and sensitive method for sex identification by amplifying gender-specific sequences, which results in different size PCR products depending on the gender of donor. Latest technology uses ion-pair reversed-phase high-performance liquid chromatography (IPRP HPLC)-DNA chromatography could identify sex at the molecular level [1]. Clifton and Rodriguez identified a sex-linked marker that could be used to identify the sex of Chinook salmon (*Oncorhynchus tshawytscha*) [2].

Information on sexual demographics of a fish's status, such as sex ratio and age-or size-at-maturity is needed to accurately assess the reproductive potential of a population. The standard method for fishes is to perform histological analysis of gonads collected during fishery census. Some of the standard test and end point and more recently identified biomarkers are gonad somatic index (GSI); histopathology, general blood chemistry and molecular measure of endocrine balance. GSI changes can be indicative of reproductive success. Significant variation in gonadal size can be measured throughout a reproductive cycle for many species and can be an indicator of reproductive maturity. This parameter is not specific to a particular mechanism of action, and may reflect a variety of factors like seasonal cycle. The GSI parameter provides a guide to comparative reproductive status. However, this technique is difficult to perform and fish must be sacrifice, and if the organisms are rare or endangered or expensive like the Giant grouper, it is not suitable to be used. Because of these reasons, researcher should work on establishing non-gonadal markers for sex and maturity.

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Received June 15, 2016; **Accepted** August 05, 2016; **Published** August 12, 2016

Citation: Daud Om A, Jasmani S, Sung YY, Bolong AA (2016) Use of Vitellogenin as Biomarker Indicator in Sex Identification of Giant Grouper (*Epinephelus lanceolatus*). Poult Fish Wildl Sci 4: 163. doi:10.4172/2375-446X.1000163

Despite that technique, gonadal steroids have been used to evaluate the sex identification. Typically, these are the reproductive steroid hormones in part because there are commercial kits available for these assays. In female fish, the important steroid hormones are estradiol (E2) and testosterone (T) while in males, the important hormones are Testosterone (T) and 11-keto-testosterone (11KT). The concentration of these steroids hormones in plasma varies according to the natural reproductive cycle of the fish. Chu-Koo, et al. had established used levels of 11KT to distinguish females from males in Paiche or Pirarucu (Araipama gigas) [3]. A useful measure of the ratio of the hormones also can be used for sex identified. Females are expected to have an E2/T ratio greater than 1, and males are expected to have a ratio less than 1. Study by Gross et al. on loggerhead sea turtles (Caretta caretta) was used E2/T ratio to distinguish between male and female hatchling [4].

Vtg, the hepatically-synthesised precursor to egg-yolk, could be an easily identifiable marker for the onset of maturation in female fish (Idler et al. 1981: Le Bail and Breton, 1981) and proposed as indicator in the plasma of maturing female fish. Immunoassay for Vtg has been developed and validated in Asian sea bass, Lates calcarifer Fazielawanie, et al. [5] tilapia, Oreochromis mossambicus Kishida and Specker [6] and European seabass, Dicentrachus labrax Mañanos et al. [7] striped bass, Morone saxatilis [8,9] channel catfish, Ictalarus punctatus Goodwin et al. [10] white-spotted char, Salvelinus leucomenis, Kwon et al. [11] Sole, Solea vulgaris [12]. Several researchers believed that Vtg could be an easily identifiable marker for onset of maturation in female fish [11-13]. Vtg can be easily collected from the plasma of maturing female and also easily purified for further analysis. This makes it ideal for the development of immunoassays for detection.

The term "antibody production" has both general and specific meanings. In the broad sense, it refers to the entire process of creating a usable specific antibody, including steps of antigen preparation, immunization, collection, screening, purification, and labelling for direct use in a particular method. In the more restricted sense, antibody production refers to the steps leading up to antibody generation but does not include various forms of purifying and labelling the antibody for particular uses. Antibody production involves preparation of antigen samples and their safe injection into laboratory, so as to evoke high expression levels of antigen-specific antibodies in the serum, which can then be recovered from the animal. Polyclonal antibodies are heterogeneous and bind to several different antigen epitopes. While decreasing the specificity and potentially increasing non-specific reactions, the polyclonal antibody is also more likely to successfully bind to the specific antigen in a variety of different test conditions.

All the above techniques for sex identification showed promise, but may have limited applicability to some species. However, identification on sex determination by using biochemical markers such as Vtg is more rapid and practical to use. In future, biochemical testing of sex gender will become practical for many hatchery operators to measure in the field or non-laboratory based analyses. Therefore, in this experiment attempts were made to discover the usefulness of Vtg as biomarker indicator on sex identification of the Giant grouper.

Materials and Methods

Induction of Vitellogenin in male giant grouper

Vitellogenin synthesis was induced in three male giant grouper (35.0 ± 2.57 kg) by injection of Estradiol-17β (E2) (Syndel Asia Sdn. Bhd.). E2 was dissolved in peanut oil: acetone (9:1) (Sigma) and stored aliquot at -30°C. Fish received intramuscular injection by 2 mg E2/kg body weight on initial day. The fish were anaesthetized by immersion in a 50 mg/l solution of tricane methane sulfonate (MS-222, Sigma-Aldrich) before taken the blood from the afferent filamentary artery (AFA) in the gill filament by heparinized needles (21G) into 1 ml plastic syringes and transferred into cryovials containing heparin (32.9 IU) and aprotinin (C 0.132 TIU, 50 µl/2.5 ml of blood). Samples were allowed to clot at 4°C overnight and plasma was separated by centrifugation at 14,000 rpm for 15 min at 4°C and keep storage at -30°C until analysis.

Gel Electrophoresis

Vtg fractions were determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis Laemmli, in 6.5% gel [14]. Five microliters of thawed plasma with concentration of 1.0 mg/ml protein from both experimental and control fish are loaded onto a 6.5% polyacrylamide SDS-PAGE, were diluted in 0.125 M Tris base, 6.5% SDS, 20% glycerol, 10% mercaptoethanol (sample buffer) and boiled for 4 min. Electrophoresis was conducted using 6.5% gels in 25 mM Trisbase, 192 mM Glycine, 0.1% SDS, pH 8.3, in polyacrylamide of at a constant voltage of 120 V for the first 15 minutes, and 150 V for the following 45 minutes. PageRuler Protein Ladder as standard was used for molecular weight determination. The gels were fixed and stained with 0.025% Coomassie Brilliant Blue. The band corresponding to the induced vtg was easily recognized by comparison of the protein banding from both control (positive-nature female and negative-nature male) fish (Figure 1).

Production steps of polyclonal antibody production

The summaries of production step for Giant grouper polyclonal antibody are in Figure 2. Followed by purification antigen, immunization of Vtg and antibody purification of polyclonal antibody. During immunization process, ELISA and Western Blot, was used for quantified and verified the development of the polyclonal antibody.

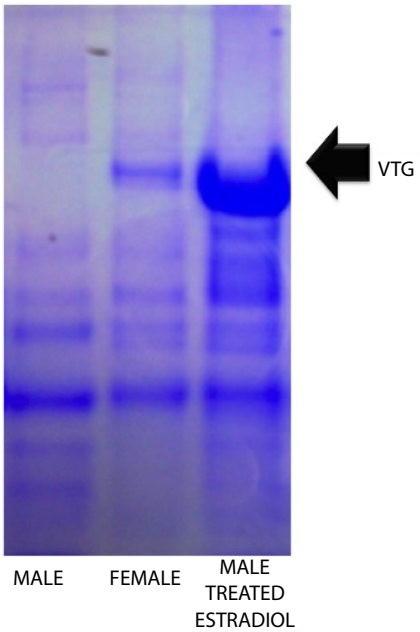

Figure 1: Denaturing (SDS) polyacrylamide gel of purified serum induction with estrogen-treated (E2) of male giant grouper serum band appeared in male giant grouper after induced with estradiol (E2).

Purification of Vitellogenin

Vitellogenin was purified using a modification of the method developed by Palumbo et al. [15]. Plasma from estrogen-treated males (induction) was pooled and used as source of vitellogenin. The plasma was fractionated on a 6B sepharose separation column (2.5 x 20 cm column).

The equilibration buffer was 20 mM Tris-HCL pH 8.0, 1 mM monothioglycerol, pH 7.5 and gradient buffer was 500 mM NaCl in equilibration buffer. Prior, plasma was diluted 1:1 in equilibration buffer and loaded onto the column. The column was washed with 30 ml of equilibration buffer to remove proteins. To know the exactly Vtg band was extracted, SDS-Page methods was done. Three ml fractions were collected throughout the procedure, and then protein levels were determined separately for each fraction utilizing a Coomassie protein assay. The fractions found to contain Vtg were pooled and dialyzed against DEAE equilibration buffer and lyophilized using dry freezer technique and was kept in frozen at -20°C prior use.

Immunization of Vitellogenin

Polyclonal antibodies against Giant grouper Vtg were generated in two New Zealand white rabbits (size 2-3 kg). The rabbit was keep in ambient temperature and acclimatize for 2 weeks before experiments started. Time line of antibody production was 72 days starting from primary immunization (Figure 2). The rabbits were immunized with 0.5 mg of purified Vtg in Freund's complete adjuvant subcutaneously. Booster immunizations were given at 30-day intervals with purified Vtg dissolved in Freund's incomplete adjuvant. One week after primary immunization, the booster 1 was performed. While, 3 weeks after that, booster II immunization was made and finally Booster III was done for production bleed or about 6 weeks from beginning. Bleeding (20 ml) was performed prior to the initial and 10 days after each subsequent immunization.

The ELISA was used to test quality of antibody to determine if there are any cross-reacting antibodies to other plasma proteins. The antibody was tested starting from first bleed immunization, followed with sample after booster I, II and III and finally from last production bleed. Blood was removed from the ear vein and was then permitted to clot for 4 hour at 37°C, and then the anti-plasma was extracted by centrifugation at 2500x g for 10 min. Centrifugation was repeated and the plasma was stored at -20°C.

Antibody purification

Protein Affinity chromatography is used for the purification of polyclonal antibodies. This is mainly due to their advantages i.e. high specificity and reversible binding of antibodies. The protein A resin has a binding capacity of ~25 mg of antibodies per 1 ml of resin. The column used for the purification purpose is Tricon TM 10/10 (Column height – 12 cm and Diameter – 10 mm, from GE Life Sciences) and packed with Protein A resin Sepharose 4 Fast Flow (GE Life Sciences) at a bed height of 10 cm. The total column volume after packing is ~7.85 ml.

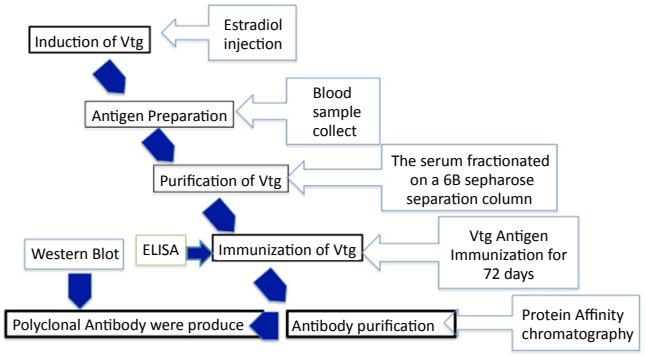

Figure 2: The production step of polyclonal antibody of Giant Grouper.

For the purification process, phosphate buffers and Glycine-HCl buffers are used for the binding and elution of polyclonal antibodies respectively. Prior to chromatography run, the plasma sample (from terminal bleeding of rabbits) was diluted in phosphate buffer to achieve the binding condition. The prepared sample was injected into the column, followed by post-load wash of binding buffer and eventually elution using Glycine-HCl buffer. Due to acidic condition of elution, the collected elate was neutralized immediately with Tris-HCl buffer. Analysis steps including column equilibration, sample loading, post-load wash, elution and re-equilibration of column are performed by AKTA Explorer System 100 (GE Life Sciences). Due to high titer of total IgG in the plasma sample, two cycles of chromatography runs were performed. Once the run was completed, the column was washed with the application buffer to equilibrate for 15 minutes. The fractions were covered and stored in 4°C refrigerator.

The efficiency of any chromatography can't be determined without performing definite analysis of targeted product. In this protein Affinity chromatography, the concentration of antibodies in the initial plasma sample was analyzed by Enzyme- linked Immunosorbent Assay (ELISA). Subsequently, the purified samples were analyzed by UV @ 280 nm for the estimation of antibodies concentration. Only the final product, i.e. sterile filtrate after formulation with respective buffer (1xPBS with 0.05% sodium azide as preservative), has extensive analysis including total IgG and Indirect ELISA.

Quantification of Vitellogenin with enzyme-linked immunosorbent assay

Quantify polyclonal antibody production was able with assay Vtg by binding it indirectly to a solid support, the surface of a microtiter well, and then detecting how much is bound. This work is applicable to the detection and semi-quantitative estimation of specific rabbit IgG antibodies. An easily-purified standard needs to be characterized so that its concentration (in µM or mg per ml) can be easily estimated by using its OD 405 nm. Quantification of Vtg is based on absorbance that was present in sample with determined through the use of a standard curve.

Two µl samples (plasma and purified Vtg) were diluted with 1998 µl phosphate- buffered saline (PBS, 136 mM NaCl, 1.5 mM KH_2PO_4, 8.2 mM Na_2HPO_4, 2.7 mM KCl, pH 7.2) to obtain a 1:1000 dilution by using a single-channel micropipette. Of this, 1 ml was added to another 2 ml of PBS (1:3,000) and 1 ml of this dilution was added to another 2 ml of PBS (1:9000). One hundred µl of the 1:9000 dilutions were added to individual wells of a polystyrene microtiter plate, and 100 µl of PBS was added to the first row as a blank (minimum 2 replicates).

Incubation took place for 1.5 hour at room temperature on an orbital shaker at 180 rpm. The plate was then washed four times with PBS. PBS blotto (5 g nonfat dry milk and 100 ml PBS) was used to block for 30 minutes at room temperature on the shaker. The plate was incubated overnight at 2-8°C with primary antibody at a dilution of 1 µ of antibody specific for Vtg (Rabbit #s, 2nd immune) to 9 µl of distilled water and 3 µl of this to 30 ml of blotto (1:100,000 dilution). The plate was washed 3 times with PBS and incubated for 2 hour on the shaker at room temperature with goat anti-rabbit immunoglobulin conjugated

to horseradish diluted 1:10,000 in PBS blotto. The plate was sealed and incubated at room temperature for 1 hour on a microplate shaker, at 180 rpm.

The plate was washed 5 times with 300 µl washing buffer. Finally, blot and vigorously bang out residual liquid over tissue paper. Followed with 100 µl substrate solution in each well. Once again, the plate was seal and incubated in the dark for 1 hour and do not shake. The reaction was stopped with 50 µl 2 M H_2SO_4. The absorbance was then read by QC-Reader-02 microplate reader at 405 nm, with reference wavelength at 492 nm, and read again 5 min later by using the microplate reader. Standard curves were run for each experiment.

Validation of Vitellogenin with western immunoblotting

Protein, separated on a gel using electrophoresis, was immediately transferred to a polyvinyl diflouride (PVDF) membrane. The gel was equilibrated in transfer buffer (25 mM Tris, pH 8.3, 192 mM glycine, 20% v/v methanol) for 15 min while the membrane was equilibrated in 100% methanol for 5 min then placed in the transfer buffer with the gel for the remaining 10 min. The proteins were transferred to the PVDF membrane under a constant voltage (100 V) electrical field for 1 hour. After the transfer, the PVDF membrane was blocked overnight at 4°C in 5% (W/V) non-fat dry milk in nanopure water (blotto; Bio-Rad cat. No 170-6404). The membrane was incubated for 1 hour with primary antibody at a dilution of 1 µl of antibody specific for vitellogenin (taken from Rabbit 2, 2nd immune) to 9 µl of distilled water and 3 µl of this to 30 ml of blocking buffer in TBS-T 0.1% tween-20 (1:100,000 dilution).

The membrane was washed two times in Tris-Tween for 10 min each and then once in Tris-saline for 5 min. The membrane was incubated for 1 hour with a goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (Bio-Rad) diluted 1:1000 in blocking buffer in TBS-T 0.1% tween-20 (Figure 3). The PVDF membrane was washed again in Tris-Tween (2 x 10 min) and Tris-saline (1 x 5 min). A sigma Fast 3.3' diaminobenzidine tetra hydrochloride (DAB) kit was used to develop the membrane (contains DAB buffered and H_2O_2) and locate the reactive proteins. After developing, the membrane was placed in distilled water to stop the reaction.

Results

Development of polyclonal antibody

The polyclonal antiplasma against Vtg was used to develop an enzyme-linked immunosorbent assay (ELISA) for Vtg. Rabbit #2 produced higher absorbance with the experimental samples showing that it had a higher titer than rabbit #1.Therefore, rabbit #2 was used in all future ELISA protocols (data not shown). Figure 4 shows the results of a Vtg ELISA using the polyclonal antibody and various dilutions of estrogen-treated Giant grouper and control (1:125 to 1:2,048,000).

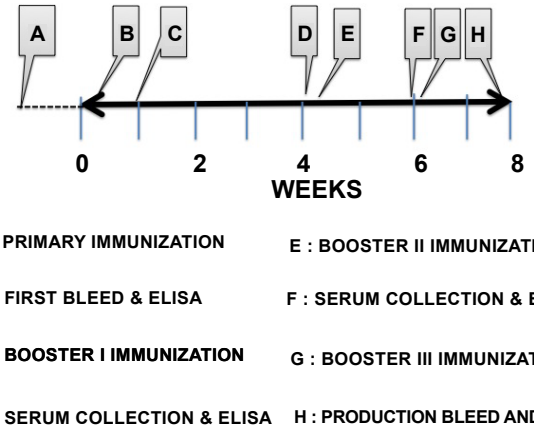

Figure 3: Project flow and time line of production of vitellogenin polyclonal antibody of giant grouper.

There was a difference in absorbance OD between two-time lines of production of Vtg polyclonal antibody of the Giant grouper. The first 2 weeks, after primary immunization (Figure 4a), the greatest difference in absorbance range from the 1:250 dilutions to the 1:8,000 dilutions. The optimal antibody dilution for the assay was determined to be 1:8,000 and was chosen as the standard dilution for the ELISA protocol. The primary antibody was used at a dilution of 1:8,000. However, after booster 1 and 2 (Figure 4b), there is no increased absorbance OD in titer of Booster 2, compared with Booster 1, absorbance OD in Booster 1 and 2 positive was higher at 1:32,000. Primary immunization at 1:512,000 and 1:2,048,000 were omitted, absorbance OD was already negative at 1:128,000 and therefore had no impact on results.

From UV@280 nm, the concentration of anti-vitellogenin antibodies in pool elute of two cycles is 4.8 mg/ml in the total volume of 40 ml with the step recovery of~84%. After formulation, the product will be delivered at the same concentration of ~5 mg/ml but in aliquots of 10 ml per tube.

Validation of Vitellogenin

In order to evaluate the anti-Vtg polyclonal, positive (estrogen-treated) and negative (untreated) Giant grouper plasma samples were

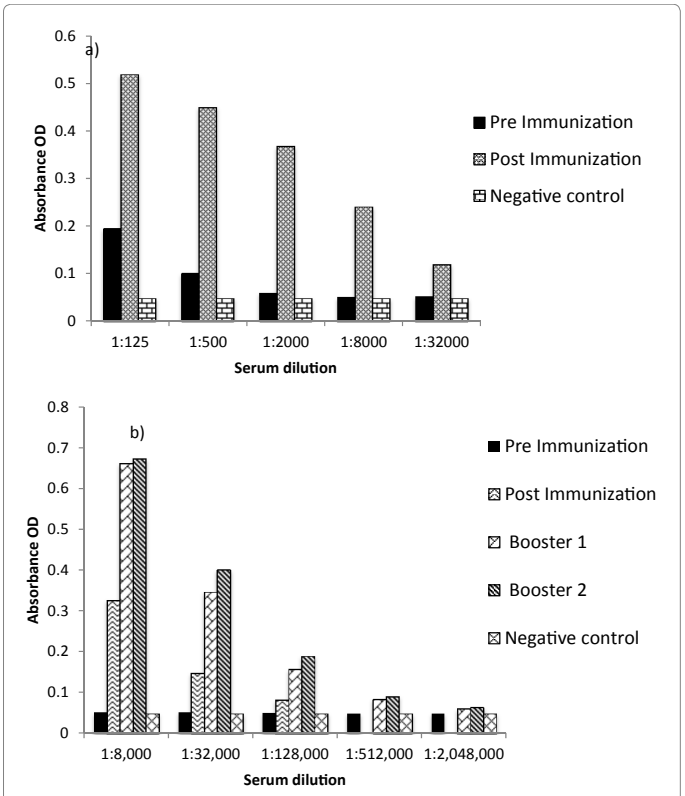

Figure 4: Vitellogenin ELISA of estrogen-treated giant grouper serum at various dilutions ranging from 1: 125 to 1:2,204,800. Specific IgG Titer of rabbits immunized with vtg: a) Primary immunization vs. Pre-Immunized Controls. b) Control vs. Primary Immunization vs. Booster 1 vs. Booster 2.

analyzed using western blot to determine if the antiplasma was able to bind to the Vtg. Figure 5 shows the SDS-PAGE revealed that the samples of estrogen-treated and female naturally contained Vtg band, while the untreated samples, male naturally did not show any Vtg band. Specificity of the antibodies was confirmed by Western blot, as shown in Figure 6 Western blot recognized the protein indicating specific binding of the Vtg antiplasma and showed reaction clearly happened in estrogen-treated and natural female but not in natural male as similar as in SDS-PAGE results.

Discussion

Several methods have been developed for the purification of Vtg, ultracentrifugation, precipitation with dimethylformamide Ansari et al. [16] selective precipitation with Mg2+-EDTA and chromatography [17]. In this study, Giant grouper Vtg was purified from plasma of E2 treated using a double chromatography method. Similar procedures have been successfully used for the purification of Vtg in *Anguilla japonica* Hara et al. [18] and *Anguilla anguilla*.

The production of polyclonal antibodies remains a common research procedure involving vertebrate animal use. Rabbits are the most commonly used laboratory animals for the production of polyclonal antibodies. The rabbit offers many advantages over other species. The adequate body size of the rabbit and the ready accessibility of the marginal ear vein and central auricular artery afford a technically easy procedure for the collection of large blood samples. Polyclonal antibody production is essential in research activity and rabbits continue to serve as one of the primary species used in polyclonal antibody production. Study by Zhang and Nie, was successful to develop polyclonal antibody of Mandarin fish (*Sinipercha chuatsi*) [19]. The antisera reacted strongly with the heavy chains of *S. chuatsi* immunoglobulin. Humoral immune responses of the mandarin fish can then be examined using the developed polyclonal antibody. Nilsen et al. was developed of quantitative vitellogenin-ELISA of common carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), Zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) [20]. They found a better sensitivity in competitive assays with polyclonal antibodies for rainbow trout and zebrafish. Similarly, finding from this experiment could be the first achievement has been made for produced Vtg polyclonal antibody of the Giant grouper.

Immunoblotting (Western blotting) is a technique, which provides information not only on antibody-antigen binding, as do ELISA and RIA procedures, but also on the electrophoretic migration and numbers of electrophoretically distinct antigens in a sample. Thus, the specificity of antibodies can be productively examined in purified and complex mixtures by this method. There are a number of techniques was developed for the measurement of Vtg in the blood of fish. Plasma calcium and protein-bound phosphate have been used as indirect indices of Vtg [21]. Immunoassays have been also developed, including radial immunoagglutination (Le Bail and Breton, [22] rocket immunoelectrophoresis and radioimmunoassay [23]. The ELISA was employed in this study and success to measure Vtg in specific IgG titer of rabbits immunized.

Other reports on the measurement of Vtg by ELISA include the white spotted char *Salvelinus leucommaenis* Kwon et al. [11] sole Sole vulgaris, brown trout Salmo trutta Maisse et al. [24] and channel catfish Ictalurus punctatus [25].

There are several types of ELISA that can be performed, including a direct assay (plasma containing Vtg is absorbed directly to the microtiter plate), a competition assay (a known amount of control Vtg adsorbed to the plate competed with Vtg) in plasma samples for antibodies, and a sandwich assay (antibodies are adsorbed to the plate and they bind Vtg in plasma). The idea behind this assay is to quantify Vtg by binding it directly to a solid support, the surface of a microtiter well, and then detecting how much is bound with specific antibody to Vtg.

A potential disadvantage of Vtg ELISA is that, depending on the particular anti-plasma or antibody employed, it can be relatively species-specific. The immunological and structural features of Vtg can vary considerably in fishes, even among species in the same family [26-28]. This diversity can require development of a new Vtg assay for each species or genera of interest, which is time-consuming and costly, but still feasible for researchers working on a single or limited number of species.

The Western blot is a variation of enzyme-immune assays. Antigen is electrophoresed through an acrylamide gel and the proteins are separated according to their size. It is blotted, to allow the transfer of

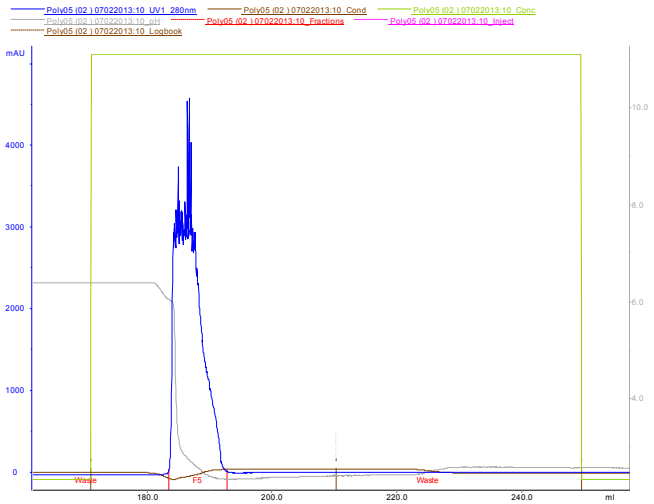

Figure 5: The chromatogram of purification using giant grouper vitellogenin serum.

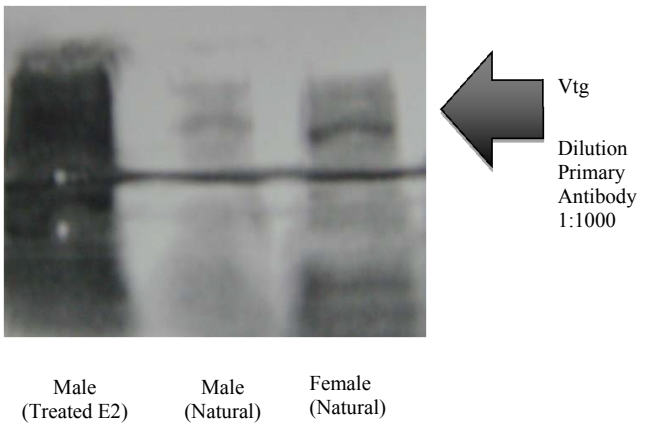

Figure 6: Western blot showing immunoreactivity of polyclonal antivitellogenin rabbit serum to estrogen-treated (E2) and control (c) giant grouper male serum. Serum were separated using denaturing polyacrylamide gel electrophoresis and transferred to a PVDF membrane for immunostating using rabbit anti-giant grouper vitellogenin serum as the primary antibody and goat anti-rabbit IgG coupled to horseradish peroxidase as the secondary antibody.

proteins to a nitrocellulose matrix that is processed to visualize the reaction. The western blot can be used to detect antibodies directed against Vtg. Western blotting is used extensively in research to determine the presence of specific proteins, to quantify their expression levels, and to determine whether they have undergone genetic or post-translational modifications. This method categorically identifies proteins of interest based on two distinguishing features: molecular mass and antibody-binding specificity.

Western blotting provides information on the identity, size and quantity of proteins. This information is useful for many applications such as disease diagnosis, agriculture, and biomedical research. Western blot was confirmed Vtg and it's could qualified by polyclonal antibody and validated back with positive control (natural female) and negative control (natural male). It showed that the anti-Giant grouper Vtg polyclonal antibody recognized the purified Giant grouper Vtg in naturally female and treated estradiol male. This reactivity demonstrates that Vtg polyclonal from immunization were highly antigenic epitopes.

Maltis and Roy, demonstrated shorthead redhorse and copper redhorse Vtg was successfully detected by using Western blot technique [29]. They used carp anti-Vtg polyclonal antibody to recognize the purified redhorse Vtg. Antibodies developed against carp Vtg have demonstrated good cross-reactivity with Vtg of cyprinids also report by Tyler et al. 1990.

Report on the development of Giant grouper Vtg polyclonal not well documented and need for extensive study to clarified. This study was the first achievement on polyclonal development of Vtg. It can be revealed that Vtg is clearly capable to be used as a biomarker indicator in sex identification of the Giant grouper. In future, polyclonal antibody can be used for developed a rapid kit sex gender identification.

The concept for developing rapid kit sex gender identification is can be explained similar to home pregnancy test method (human consume) involved an elegant application of polyclonal antibody technology to detect the human chorionic gonadotropin (HCG), which is produced by the developing embryo. The test is simple to carry out; a few drops of urine are placed in the sample window, and the result is shown within five minute. The additions of the urine solubilize a polyclonal antibody for HCG, which is covalently bound to tiny blue beads. A second monoclonal, specific for another region of the HCG molecule, is firmly attached in a line at the result window. If HCG is present in the sample it is bound by the first antibody, forming a blue bead-antibody-HCG complex. As the urine diffuses through the strip, any HCG present becomes bound at the second antibody site and this concentrates the blue bead complex in a line, a positive results. A third antibody recognizes the constant region of the first antibody and bind the excess, thus providing a control to show that sufficient urine had been added to the test strip, the most likely form of error.

Application of rapid kit for Giant grouper sex detection can be much more easily and quick. The skin mucus of fish could be similar to the urine, source of HCG in home pregnancy test method (human consume) rather than blood serum sample. Study by Gordon et al. determined Vtg in skin mucus specimen from Coho salmon (*Oncorhynchus kisutch*) [30]. The concentration of Vtg in mucus has increased when female gonad weight was in between 1 to 6% of body weight, 4-5 months prior to spawning [22,31-34]. Therefore, a simple, rapid, non-interactive technique for sex determination of Giant grouper that can be used by fish farmers and hatchery operators may be possible [35-38].

Conclusion

From discussion above, it can be revealed that polyclonal antibody of giant grouper is success to produced and accumulations of estrogen responsive proteins associated with exposure to estrogenic hormones were positively effect and can quantified with ELISA and validate by using western blot technique.

Acknowledgements

This study is funded by the Ministry of Science, Technology and Innovation, Malaysia under Intensified Research in Priority Areas (E-Science Fund, 004-07-05-06).

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