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A monoclonal antibody against *Lates calcarifer* vitellogenin and a competitive ELISA to evaluate vitellogenin induction after exposure to xenoestrogen

Witchuda Prasatkaew[1], *Phochit Nanthanawat*[2,*], *Nanthika Khongchareonporn*[3], *Sutin Kingtong*[4]

1. *Environmental Science program, Faculty of Science, Burapha University, Chon Buri 20131, Thailand*
2. *Department of Biotechnology, Faculty of Science, Burapha University, Chon Buri 20131, Thailand*
3. *The Institute of Biotechnology and Genetic Engineering, Chulalongkorn University, Bangkok 10400, Thailand*
4. *Department of Biology, Faculty of Science, Burapha University, Chon Buri 20131, Thailand*

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ABSTRACT

A monoclonal antibody specific to sea bass (*Lates calcarifer*) vitellogenin (VTG) was developed, for use as a tool for monitoring endocrine disrupting chemicals (EDCs). VTG was induced in sea bass by intramuscular injection of 17β-estradiol (E_2: 2 mg/kg) every three days. Blood was collected three days after the last injection. Plasma VTG was then purified by chromatography in hydroxyapatite and a sephacryl-S300 column. Characterizations of purified VTG were done by phospholipoglycoprotein staining on a native-PAGE with confirmation by mass spectrometry (LC-MS/MS). Antibody was raised in mice by injection of purified VTG. After monoclonal antibody production, the hybridoma clone No. 41 (MAb-sea bass VTG 41) was selected and developed for quantification of VTG by competitive enzyme-linked immunosorbent assay (ELISA). The ELISA method was sensitive with a detection limit of VTG 40 ng/mL. MAb-sea bass VTG 41 was specific to VTG from E_2-treated sea bass and others EDCs (Nonylphenol, Benzo[a]pyrene and $CdCl_2$). Moreover, cross-reactivity was also found in E_2-treated coral grouper (*Epinephelus corallicola*). The ELISA method obtained from this work can be further applied for the assessment of EDCs in Thailand and Southeast Asia's aquatic environment.

Introduction

Vitellogenin (VTG) is an accepted biomarker for estrogenic endocrine disrupting chemicals (EDCs), a group of substances that interfere with the endocrine system, due to their specificity for estrogen. VTG is a major serum phospholipoglycoprotein synthesized in the liver of female oviparous vertebrates and transported to the ovary via the bloodstream. VTG is taken up by the oocyte and cleaved into yolk proteins, lipovitellin and phosvitin, nutrients for oocytes and embryos (Hiramatsu et al., 2002; Maltais and Roy, 2009; Scott and Robinson, 2008). VTG is normally synthesized in mature females in response to endogenous estrogen in contrast to males and juveniles where the latter is low to undetectable (Dang, 2016; Matozzo et al., 2008). However, males and juveniles also have the VTG gene, and estrogen receptors (ER) males and juveniles provide

* *Corresponding author. E-mail:* phochit@buu.ac.th (Phochit Nanthanawat).

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evidence of ECD exposure making it a useful biomarker of prior exposure (Booth and Skene, 2006; Maltais and Roy, 2009).

Techniques for VTG detection were developed for studies on endocrine disruption in several vertebrates (Selcer and Verbanic, 2014). Currently, antibodies against VTG have been developed for detection in specific marine and freshwater fish (An et al., 2008; Booth and Skene, 2006; Van den Belt et al., 2003). Species-specific binding of antibodies suggests relevant, heterologous antibodies from genetically dissimilar species are may be less sensitive than homologous antibodies from indigenous and genetically similar species (Wang et al., 2015). For that reason, existing antibodies produced against VTG are not useful in Thailand and Southeast Asia due to the lack of availability of those fish and low species-specific binding of antibodies. We therefore produced monoclonal antibody against VTG and develop detection technique in a native fish. In Southeast Asia a suitable candidate is sea bass, *Lates calcarifer*, a euryhaline and important economic species in the wild and cage culture fisheries.

The purpose of the present study was to produce a monoclonal antibody against VTG in sea bass and to develop an enzyme-linked immunosorbent assay (ELISA) for further detection and monitoring of EDCs in Southeast Asia.

1. Materials and methods

1.1. Chemicals

17β-Estradiol (E_2), Methyl Green, Sudan Black B, and Alcian Blue were purchased from Sigma-Aldrich (Thailand). Other reagents for native polyacrylamide gel electrophoresis (native-PAGE), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot, including molecular weight standard, nitrocellulose membrane, Coomassie brilliant blue R-250 (CBR-250) and hydroxyapatite (BioGel-HPT) were obtained from Bio-Rad (Thailand). Sephacryl™ S-300 was from GE Healthcare (Thailand). All selected EDC chemicals (Nonylphenol; NP, Benzo[a]pyrene; BaP and Cadmium chloride; $CdCl_2$) were obtained from Sigma-Aldrich (Thailand), with a purity of 98.0%, 96.0% and 99.0%, respectively.

1.2. Animal husbandry

One-year-old sea bass (233 ± 64.61 g, n = 15) were used for VTG induction; they were provided by Talaythong farm, Chon Buri, Thailand and were maintained in seawater (25–28°C) tanks (5000 L).

Juvenile sea bass (13.45 ± 1.00 g, n = 100) used in the VTG induction via EDCs were purchased from Pramote farm, Chon Buri, Thailand. Fish were maintained in aerated freshwater tanks (100 L).

Cross-reactivity of hybridoma clone No. 41 (MAb-sea bass VTG 41) was tested against eight indigenous freshwater and marine species. These include two sub-species of Nile tilapia *Oreochromis niloticus* (206.75 ± 24.78 g, n = 3) and *O. niloticus-mossambicus* (206.75 ± 24.78 g, n = 3), silver barbs *Barbonymus gonionotus* (55.77 ± 11.15 g, n = 3), hybrid catfish *Clarias macrocephalus* × *C. gariepinus* (250.77 ± 6.93 g, n = 3), striped snakehead *Channa striata* (506.75 ± 21.33 g, n = 3), snake skin gourami *Trichogaster pectoralis* (46.75 ± 4.01 g, n = 3), and moonbeam gourami *T. microlepis* (42.77 ± 4.40 g, n = 3). Coral grouper *E. corallicola* (92.00 ± 7.81 g, n = 3) was also selected for cross-reactivity testing as a sentinel marine species. They were kept in 500-L aerated freshwater tanks whereas coral groupers were held in sea water. All freshwater fish were obtained from the department of fisheries, faculty of agriculture and technology, Rajamangala University of Technology Isan, Surin Campus, and coral groupers were provided by Trad Coastal Fisheries Research and Development Center.

All fish were held in the laboratory for 7 days. Water was replaced at 30% volume every three days. Fish were fed with commercial fish feed (Profeed, Thai Union Feedmill, Thailand) once a day.

The experimental protocol for the animal study was approved by the Burapha University Institutional Animal Care and Use Committee (approval number 2/2557, date of approval September, 2015).

1.3. VTG induction in sea bass

VTG synthesis was induced by an intramuscular (I.M.) injection of 17 β-estradiol (E_2). All treated fish were injected with 2 mg E_2/kg body weight dissolved in dimethyl sulfoxide (DMSO) and 0.15-mol/L phosphate buffer saline (PBS) pH 7.4. Control fish were injected with the same volumes of DMSO and PBS without E_2 (vehicle control). Sea bass were given three I.M. injections at an interval of 2 weeks, thereafter a blood sample from which a plasma sample was stored at −80°C.

1.4. Purification of sea bass VTG

Plasma VTG from E_2-treated fish was purified via a two steps chromatographic process, with hydroxyapatite (BioGel-HTP; Bio-Rad, Thailand) in the first column and sephacryl S-300 (GE Healthcare, Thailand) in the second (Thanomsit et al., 2009). Fractions containing VTG were stored at −80°C.

1.5. Characterization of VTGs

The purified VTG was run in 7.5% native-PAGE with 4% stacking gel in a Mini protein® Tetra cell apparatus (Bio-Rad, Thailand). Distribution pattern of purified VTGs was visualized by 0.1% CBR-250 staining. Phosphorus, lipid, and carbohydrate in VTG gels was detected by staining with 0.5% Methyl Green (Cutting and Roth, 1973), 0.5% Sudan Black B (Prat et al., 1969) and 0.1% Alcian Blue (Zacharius et al., 1969). Purified VTGs were separated in 7.5% SDS-PAGE (Laemmli, 1970). After separation, protein gel bands were visualized by 0.1% CBR-250 staining. Prestained SDS-PAGE standards broad range (Bio-Rad, Thailand) was run in the gel in order to determine the molecular weight of VTG and further used for Western Blot.

1.6. Protein identification by LC–MS/MS and bioinformatics search

VTG were confirmed with LC–MS/MS and database comparison to identify proteins. Protein analysis was modified from Srisomsap et al. (2010). Briefly, protein bands were cut from SDS-PAGE and twice immersed in a solution of 25-mmol/L ammonium bicarbonate in 50% acetonitrile and protein reduced and alkylated in 10-mmol/L dithiothreitol (DTT) and

100-mmol/L iodoacetamide, respectively. Gel pieces were incubated in trypsin (Promega, USA) overnight. Digested peptides were analyzed via a ESI-Q-TOF mass spectrometer. MS/MS files were used to search against the SwissProt database (searching was performed on January 2017), using the Mascot algorithm (http://www.matrixscience.com).

1.7. Production of monoclonal antibody

Female BALB/cMlac mice (six weeks of age, National Laboratory Animal Centre, Mahidol University, Thailand) were I.P. injected with purified VTG emulsified in complete Freund's adjuvant, with booster injections of emulsion with incomplete Freund's adjuvant at two week intervals three times plus an injection three days before fusion. The immunized mice were killed and the spleen removed. Spleenocytes were fused with P3X using polyethylene glycol (50% PEG, W/V) and selected by HAT medium (Khamjing et al., 2011). During culture, positive hybridomas were screened, subcultured, and recloned. The supernatant was collected and clones were stored in liquid nitrogen.

1.8. Western blot

Western Blot was used to test specificity and cross reactivity of MAb. Protein samples were separated by 7.5% SDS-PAGE and transferred to the nitrocellulose membrane (Towbin et al., 1979). Accordingly, the membrane was incubated with MAb at 4°C overnight. After three washes in PBST (0.15 mol/L PBS solution, pH = 7.4 containing 1% Tween-20), the membrane was incubated in secondary antibody (goat anti-mouse IgG–HRP conjugate (GAM-HRP), 1:3000) as secondary antibody for 2 hr at room temperature. Then, the membrane was washed again and stained with a chromogenic substrate solution (0.03% 3,3′-diaminobenzidine tetrahydrochloride (DAB), 0.06% H_2O_2, and 0.05% $CoCl_2$ in 0.15-mol/L PBS, pH = 7.4).

1.9. Development of a competitive ELISA

Competitive ELISA was used to optimize the measurement of plasma VTG following the protocol by Lomax et al. (1998) with minor modifications. Purified VTGs diluted in blocking solution (5% skim milk in 0.15-mol/L PBS, pH = 7.4) was applied to the inner surface of each well (100 μL/well) and incubated at 4°C overnight. In parallel, a pre-incubation step was performed by adding MAb-sea bass VTG 41 (1:500) with the dilution of purified VTG (0.04–40,000 ng/mL) at 4°C overnight. After coating, well plates were washed three times with PBST, and 200 μL of blocking solution was added and incubated for 1 hr at room temperature. Then, 100 μL of each pre-incubated solution was dispensed in duplicate into 96-well plates, and incubated for 2 hr. After washing, GAM-HRP (1:10,000) was added and incubated for 2 hr. Finally, 100 μL of chromogenic substrate solution containing of 0.025% 3,3′,5,5′-tetramethylbenzidine (TMB), 2.5% DMSO and 0.35% H_2O_2 in 0.1-mol/L citrate buffer, pH = 4.5, was added and incubated for 15 min. The reaction was stopped by adding 100 μL of 1 mol/L H_2SO_4 to each well. Absorbance (450 nm) was measured using a microtiterplate reader (Versamax, USA). Standards and samples were run in duplicate.

1.10. VTG induction in juvenile sea bass via EDCs

After being held in the laboratory for 7 days, juvenile sea bass were separated into five groups consisting of 20 sea bass each, one control and four treated groups ((1) NP 25 mg/kg, (2) BaP 0.5 mg/kg, (3) $CdCl_2$ 0.5 mg/kg, and (4) E_2 2 mg/kg). Each chemical was dissolved in DMSO (0.7%, W/V) and 0.15 mol/L PBS, pH = 7.4. For control group, sea bass were injected with the same volumes of DMSO and PBS. Fish were given I.P. injections with each treatment after which plasma was collected from the caudal vein every three days for six intervals and immediately centrifuged at 1500× *g* for 5 min. Competitive ELISA was used to determine VTGs in plasma.

1.11. Cross-reactivity of MAb-sea bass VTG 41

Eight fish species (*O. niloticus*, *B. gonionotus*, *C. macrocephalus* × *C. gariepinus O. niloticus–mossambicus*, *C. striata*, *T. pectoralis*, *T. microlepis*, and *E. corallicola*) were selected as sentinel freshwater and marine species to tested cross-reactivity of obtained MAb. Fish were given a series of I.M. injections with E_2, the same condition as *L. calcarifer*. At the end of the experiment, blood was collected. Plasma was separated and used for cross-reactivity testing by Western Blot.

1.12. Statistical analysis

Plasma VTG between non-E_2-treated group (control) and E_2-treated group were compared by independent-2-sample *t*-test. Difference between means of various fish treatment groups were determined by analysis of variance (ANOVA) and means were compared using Tukey pairwise comparisons with minitab® 17 software (entitlement i.d.: 2ec6-9b37-1508-0264-2c55-c33).

2. Results

2.1. Induction and purification of sea bass VTG

Protein concentrations in plasma of E_2-treated sea bass increased significantly ($P < 0.05$) from (27.50 ± 6.26) mg/mL (mean ± SD) in non E_2-treated fish to (45.61 ± 8.34) mg/mL in E_2-treated fish. VTG was purified by combinations of anion exchange chromatography on hydroxyapatite followed by gel filtration using sephacryl S-300. When plasma from non E_2-treated and E_2-treated sea bass were loaded on a hydroxyapatite column the elution profile for the latter displayed a prominent peak relative to that for the former at fraction 83 of E_2-treated, showing that it was induced by E_2 (Fig. 1). This peak was subjected to gel filtration chromatography.

A single difference peak was obtained from sephacryl S-300 column (Fig. 1b). The protein concentration of this peak was 0.90 mg/mL.

2.2. Characterization of VTG

Purified proteins showed VTGs characteristics in their molecules by the staining of phosphorus, lipid, and carbohydrate

on a 7.5% native-PAGE (Fig. 2). SDS-PAGE and Western Blot showed that the major band of 169 kDa was largely increased in plasma of E_2-treated fish (**Lane 2** in Fig. 3a and b) compared to non-E_2-treated fish (**Lane 1** in Fig. 3a). This protein band had identical molecular weight to protein obtained from fraction 83 of hydroxyapatite column (**Lane 3** in Fig. 3a and b) and the protein fraction obtained from sephacryl S-300 column (**Lane 4** in Fig. 3a and b). In addition, minor bands could be detected with molecular weights of 132, 112, 86 and 69 kDa. A positive reaction was not detected in non-E_2-treated fish (**Lane 1** in Fig. 3b).

MS/MS analysis and database comparison confirmed that the bands corresponding to VTGs in SDS-PAGE (**Lane 4** in Fig. 3a) were matched to two VTG forms, including VTG I and VTG II as detailed in Table 1. However, band 69 kDa did not match any peptide.

2.3. Production of monoclonal antibody

Three specific VTG hybridoma clones against sea bass were selected to test sensitivity of antibody. Cell culture supernatant of the hybridoma clone No. 41 (MAb-sea bass VTG 41) showed the highest sensitivity in all immunoassay testing (data not shown). Therefore, MAb-sea bass VTG 41 was selected for mass production. This indicated optimal dilutions for Western Blot and ELISA of this MAbs were 1:10,000 and 1:500, respectively.

2.4. Development of competitive ELISA

A competitive ELISA was developed to detect plasma VTG levels of sea bass using purified VTGs and MAb-sea bass VTG 41. Optimal assay conditions were obtained by coating purified VTG at 20 μg/mL, MAb-sea bass VTG 41 and GAM-HRP as 1:500 and 1:10,000, respectively. The maximum OD values from this condition was approximately 1.73–2.22. The assay range of the standard curve was 40–4000 ng/mL. The inter- and intra-assay at 90% binding were 11.46% (n = 6) and 4.51% (n = 6), respectively. The recovery was between 12.30–91.48% (Fig. 4).

2.5. VTG induction in juvenile sea bass via EDCs

VTG concentrations were evaluated by competitive ELISA after injection with NP (25 mg/kg), BaP (0.5 mg/kg), $CdCl_2$ (0.5 mg/kg), and E_2 (2 mg/kg; Fig. 5). Mortalities occurred only among $CdCl_2$-treated fish at 21.88%. VTG levels increased significantly in E_2-treated fish on day 3. VTG levels reached a maximum on day 12 and decreased on day 15. The VTG response to injection with $CdCl_2$ was a similar pattern to that in the E_2-treated group. VTG induction reached a maximum after injection with NP and BaP at day 9 and 15, respectively. The maximum level of VTG induction from E_2-injection was higher by 5–10 fold relative to the other chemical treatments.

2.6. Cross-reactivity of MAb-sea bass VTG 41

Western Blot showed the specificity of MAb-sea bass VTG 41 to sea bass VTG and cross-reactivity to VTG from coral grouper, while no cross-reactivity was observed in other fish species (data not shown). The polypeptide bands of coral grouper VTG that display cross-reactivity with MAb-sea bass VTG 41 revealed five bands as 152, 133, 79, 70, and 63 kDa (Fig. 6).

3. Discussion

It is well established that the induction of VTG in sea bass can be artificially stimulated (Fazielawanie et al., 2013; Pan et al., 2012; Roy et al., 2004). Results showed a significant increase in total plasma protein after administration of E_2 at a dose of 2 mg/kg. The two step chromatographic purification with hydroxyapatite and sephacryl S-300 removed most of the unwanted plasma proteins.

Two bands of purified VTG were found on native-PAGE. We have demonstrated that these two bands are VTG from their lipid, carbohydrate, and phosphorus content and possibly the only two forms of VTG gene (*Vtg*) in sea bass. The results of our study are similar to those in stone flounder *Kareius bicolouratus* which also found two phospholipoglycoprotein bands with 550 and 520 kDa on 6.5% native-PAGE (Pan et al., 2012). In addition, two forms of *Vtg* have been reported in

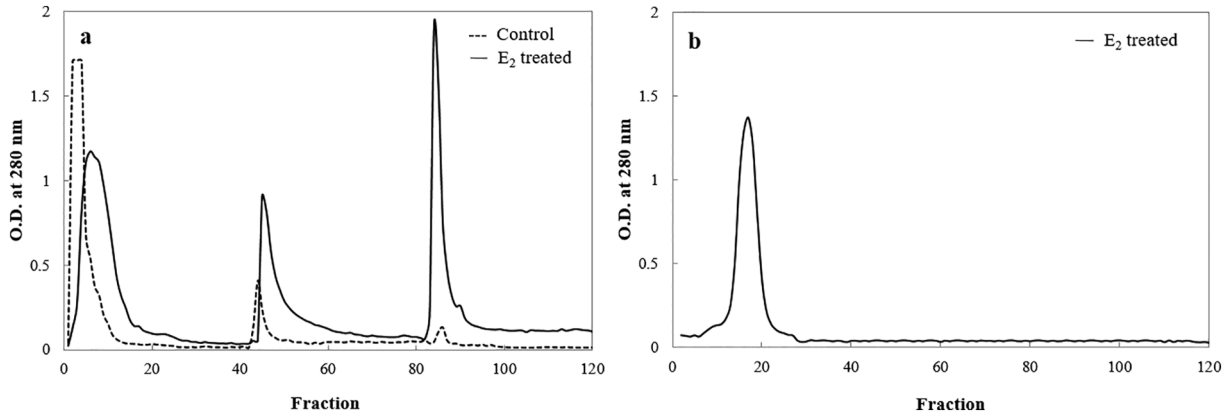

Fig. 1 – Purification of plasma from non-E_2-treated sea bass and E_2-treated sea bass by hydroxyapatite (a) and Sephacryl S-300 column (b).

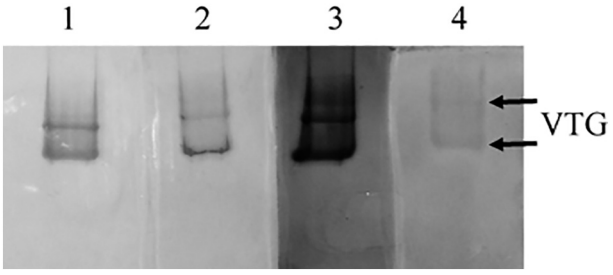

Fig. 2 – Characterization of purified VTG (vitellogenin) on 7.5% native-PAGE and stained with CBR-250 (1) Methyl Green (2), Sudan Black B (3) and Alcian Blue (4).

other fish including barfin flounder *Verasper moseri*, California halibut *Paralichthys californicus* and Chilean flounder *P. adspersus* (Leonardi et al., 2012; Matsubara et al., 1999; Palumbo et al., 2007).

Combinations of MS/MS-analysis and database search revealed all four bands (169, 132, 112, and 89 kDa) were VTG, which included two types of *Vtg* (*VtgI* and *VtgII*). Since the sea bass lacks a reference genome, identification was based on *VtgI* from rainbow trout *O. mykiss* (Swiss-Prot Accession No. Q92093) and *VtgI* plus *VtgII* from killifish *F. heteroclitus* (Swiss-Prot Accession No. Q90508 and No. Q98893). This suggests that *Vtg* gene of sea bass have more than one form. In contrast, European sea bass *Dicentrarchus labrax* possesses three types of *Vtg* including *VtgAa, VtgAb* and *VtgC* (Yilmaz et al., 2015). Furthermore, variation of *Vtg* gene number is reported in many species; for example, one or two *Vtg* genes in barfin flounder *V. moseri,* and California halibut *P. californicus* and three *Vtg* genes in white perch, *Morone americana* (Hiramatsu et al., 2002; Matsubara et al., 1999; Palumbo et al., 2007; Utarabhand and Bunlipatanon, 1996).

The monoclonal antibody (MAb-sea bass VTG 41) in the present study has shown greater sensitivity than other MAbs such as MAb-crucian carp, *Carassius carassius* VTG in mouse and PAb-sea bass VTG in rabbit (An et al., 2007; Fazielawanie et al., 2013). MAb-sea bass VTG 41 also showed specificity with plasma and purified VTG from E_2-treated fish and had no reactivity with E_2-untreated fish. Purified VTG had protein bands at molecular weight of 169, 132, 112, 86 and 69 kDa. This could be related to proteolysis after purification as in stone flounder *Kareius bicolouratus* (Pan et al., 2012). Moreover, the degradation fragments of VTG during electrophoresis or the presence of more than one form of VTG can result from the use of SDS and β-mercaptoethanol (Fazielawanie et al., 2013; Scott and Robinson, 2008). Interestingly, the barely detectable band (112 and 86 kDa) and faint band (132 kDa) in SDS-PAGE were more apparent in Western Blot, indicating a high affinity for an antibody binding site (Pan et al., 2012). As in European sea bass *D. labrax* treated with E_2 (2 mg/kg BW), a major band of 180 kDa and four minor bands were found, corresponding to breakdown products with lower molecular mass (Vaccaro et al., 2005). In addition, VTG of other fish such as shorthead redhorse *Moxostoma macrolepidotum*, Atlantic cod *Gadus morhua*, California halibut *P. californicus*, and stone flounder *K. bicolouratus* also showed similar molecular weight in the range of 100–200 kDa (Luo et al., 2011; Maltais and Roy, 2009; Palumbo et al., 2007; Pan et al., 2012; Scott et al., 2006).

We also developed a competitive ELISA for VTG quantification. This method is sensitive, with a detection limit of VTG at 40 ng/mL and a working range of 40–4000 ng/mL. Sensitivity of sea bass VTG detection by ELISA is similar to that for other species; e.g. 43 ng/mL in goldfish, *Carassius auratus* (Palermo et al., 2008), 33 ng/mL in striped bass, *Morone saxatilis* (Heppell et al., 1999), 15 ng/mL in smooth flounder *Pleuronectes putnami* (Roy et al., 2004) and 10 ng/mL in Atlantic cod, *G. morhua* (Scott et al., 2006). This sensitivity was enough to detect VTG level in feral fish, because the range of VTG in feral fish is higher than the detection limit; <0.01 μg/mL to >50 mg/mL (Scott et al., 2007). Another study reported the plasma VTG concentration in a field study of brown trout, *Salmo trutta* was >50 ng/mL to >1 μg/mL (Bjerregaard et al., 2008; Morthorst et al., 2018). Therefore, the competitive ELISA developed in the present study is suitable for monitoring EDCs in the field.

In the present study, MAb-sea bass VTG 41 detected plasma VTG, induced by EDCs. A significant up-regulation of VTG was observed in plasma of E_2-treated fish, reaching a maximum on day 12. Therefore, the optimum times to evaluate VTG expression in sea bass was not more than 12 days after induction. A similar delay in VTG maximum induction was found in European sea bass *D. labrax* and rainbow trout *O. mykiss* at 14 and 15 days, respectively (Olsson et al., 1995; Vaccaro et al., 2005). Nonylphenol, BaP, and $CdCl_2$

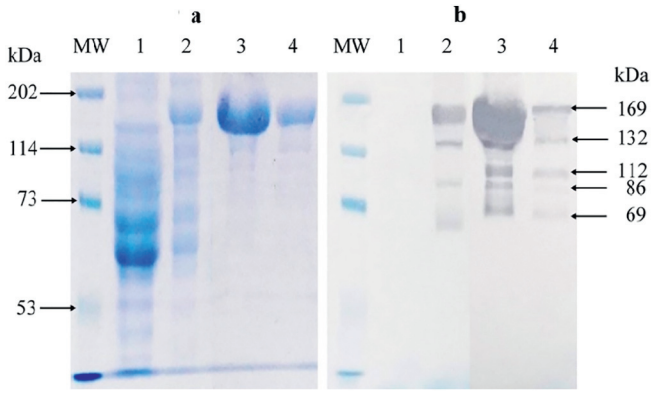

Fig. 3 – Detection of plasma VTG on 7.5% SDS-PAGE (a) and Western Blot (b) non-E_2-treated sea bass (lane 1), E_2-treated sea bass (lane 2), purified VTG from hydroxyapatite column (lane 3) and purified VTG from Sephacryl S-300 column (lane 4).

Table 1 – Details of identified protein from sea bass VTG by proteomic approach.

MW on gel (kDa)	Accession number	Gene name	Organism	Function	Score	Theoretical MW	Experimental MW	Peptide match
169	Q92093	*Vtg1*	*Oncorhynchus mykiss*	Precursor of the major egg-yolk proteins of oviparous organisms	140	183	169	R.GILNILQLNIK.K K.FFGQEIAFANIDK.S K.YEALLLGGLPEEGLAR.A
	Q90508	*Vtg1*	*Fundulus heteroclitus*	As above	93	188	169	K.FLELVQLLR.I R.GILNILQLNIK.K R.VNSTRSSSSSSR.T K.YEALILGGLPEEGLAR.A
	Q98893	*VtgII*	*Fundulus heteroclitus*	As above	63	186	169	K.FIELIQLLR.V
132	–	Fragment of *Vtg1*	*Fundulus heteroclitus*	As above	39	–	132	K.FFGQEIAFANIDK.S
112	Q92093	*Vtg1*	*Oncorhynchus mykiss*	As above	116	183	112	R.GILNILQLNIK.K K.FFGQEIAFANIDK.S
	Q90508	*Vtg1*	*Fundulus heteroclitus*	As above	68	188	112	K.FLELVQLLR.I R.GILNILQLNIK.K R.VNSTRSSSSSSR.T
	Q98893	*VtgII*	*Fundulus heteroclitus*	As above	62	186	112	K.FIELIQLLR.V R.GSLKYELGTEFLQTPIQLLR.I
86	Q92093	*Vtg1*	*Oncorhynchus mykiss*	As above	134	183	86	R.GILNILQLNIK.K K.FFGQEIAFANIDK.S

MW: molecular weight; –: no peptide matches.

are also estrogenic, as indicated by an increase in plasma VTG in comparison to controls. A similar observation was made for other fish such as European sea bass *D. labrax*, mosquitofish *Gambusia affinis*, and barfin plaice *Liopsetta pinnifasciata* (Jung et al., 2012; Hoffmann and Oris, 2006; Huang et al., 2014; Syasina and Shved, 2015; Vaccaro et al., 2005). However, some researchers have argued that induction of VTG is not altered by metal (Chang et al., 2011; Gerbron et al., 2015; Hwang et al., 2000). Cd showed inhibitory effect on VTG production when the amount of Cd added exceeded the hepatocytes' ability to synthesize metallothioneins. In conclusion, Cd has been found to interact both directly with estrogen receptor (ER) and indirectly to affect ER mediated protein levels. Low concentrations of Cd have been found to activate gene expression with high affinity for the hormone-binding domain in a manner that was inhibited by ER antagonist, further suggesting specificity in the time course of the ER response to natural or xenoestrogens in fish (Hwang et al., 2000; Tilton et al., 2003).

The MAb-sea bass VTG 41 was specific to marine fish VTGs and showed no reactivity with VTG from any of the freshwater fish examined. It is highly probable that the antibody recognized an epitope of the VTG molecule conserved in marine fish. Previous studies have shown that the VTG molecule is highly conserved within the same family but not in different families (An et al., 2007; Leonardi et al., 2012) In contrast, no cross-

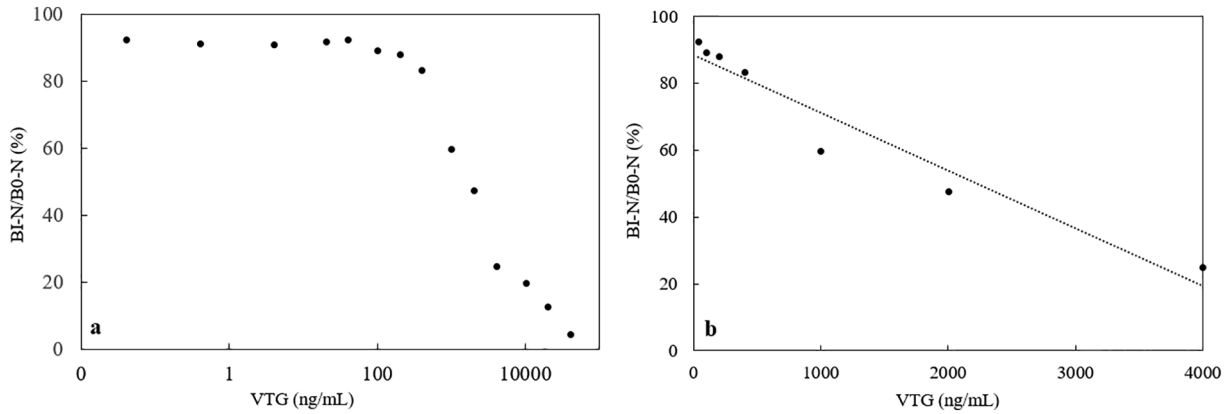

Fig. 4 – Binding curves of purified VTG (a) and standard curves of the purified VTG dilutions from 40 to 4000 ng/mL (b) by competitive ELISA.

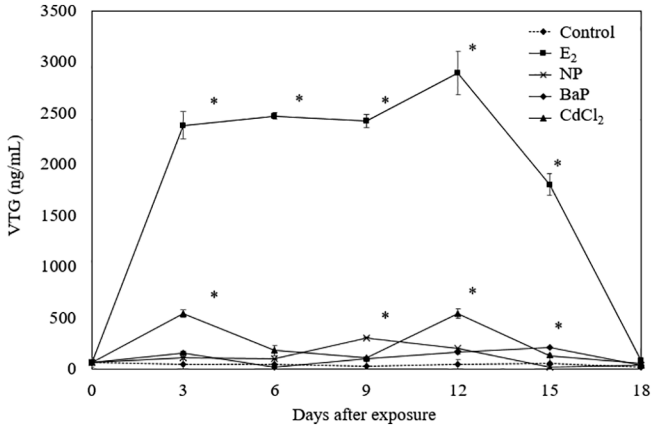

Fig. 5 – Profile of plasma VTG levels from control and treated fish determined by competitive ELISA. Values are mean ± SE (n = 3). An asterisk indicates a significant different from controls (p < 0.05) determined by ANOVA.

reactivity was noted between PAb anti-Atlantic salmon *S. salar* VTGs and either rainbow trout *O. mykiss* or greenback flounder *Rhombosolea tapirina* VTGs (Watts et al., 2003). However, some studies have shown VTG cross-reactivity between a few or even many non-related species, suggestive of a MAb-rainbow trout VTG 2D8 (Heppell and Sullivan, 1999). For example, the PAb rabbit anti-turbot *Scophthalmus maximus* VTG (Biosense Laboratories, Bergen, Norway), have shown the cross-reactivity with VTG from the Chilean Flounder, *P. adspersus* (Leonardi et al., 2012).

In conclusion, the present study indicates that there are at least two VTG genes in sea bass with four major bands in denatured form using Western Blot at 169, 132, 112, and 86 kDa. The MAb-sea bass VTG 41 was effective to detect and monitor VTG in sea bass following exposure to estrogen and xenoestrogen. Moreover, the cross-reactivity of this antibody was effective also in the coral grouper. Our study generated suitable tools, a sentinel species for assessment of VTG induction, and can be applied in the field for study for environmental monitoring in Southeast Asia and beyond.

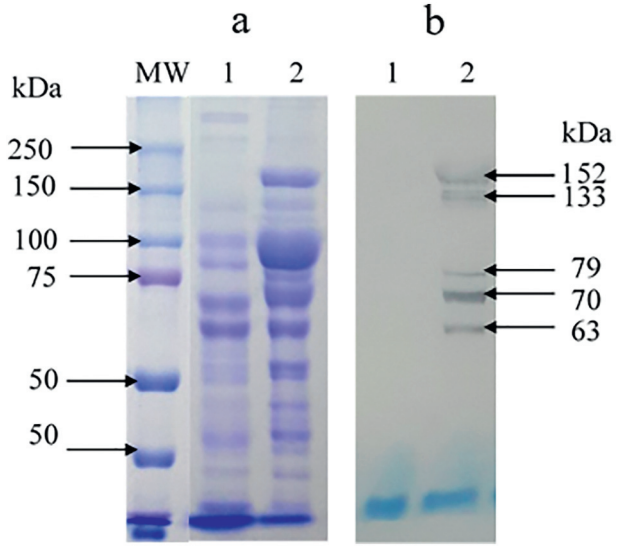

Fig. 6 – SDS-PAGE (a) and Western Blot (b) of plasma from non-E_2-treated (1) and E_2-treated coral grouper (2). MW is Pre-stained plus SDS-PAGE standard, Broad Range (Bio-Rad).

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