



# Development of homologous enzyme-linked immunosorbent assays to quantify two forms of vitellogenin in guppy (*Poecilia reticulata*)

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## Abstract

Guppy (*Poecilia reticulata*) is a promising model organism in toxicological studies, and vitellogenin (Vtg) is a commonly used biomarker for environmental estrogens. Although an ELISA for guppy Vtg has been developed previously, we found that guppy had two forms of Vtgs. In this study, two Vtgs were characterized and enzyme-linked immunosorbent assays (ELISAs) for each Vtg were developed. Two Vtgs purified from 17 $\beta$ -estradiol (E<sub>2</sub>)-exposed guppy were characterized as phospholipoglycoproteins with molecular weights of ~ 520 and ~ 480 kDa, respectively. In SDS-PAGE, one purified Vtg appeared as three major bands of ~ 210, ~ 126, and ~ 102 kDa, and the other revealed a clear band of ~ 68 kDa. Matrix-assisted laser desorption/ionization–time of flight/time of flight mass spectrometry analysis showed that they were VtgAb and VtgC. Using purified Vtgs and their corresponding antibodies, two sandwich ELISAs with working ranges of 7.8–1000 and 15.6–500 ng/mL were developed. Precision tests showed that intra- and inter-assay coefficients of variations of both ELISAs were below 10%. Parallelism between Vtg standard curves and serial dilutions of whole body homogenate from E<sub>2</sub>-exposed guppy confirmed that two ELISAs could quantify guppy Vtgs. Furthermore, two ELISAs were used to measure Vtg inductions in liver, caudal fin and whole body of male guppy exposed to 17 $\alpha$ -ethinylestradiol to validate their use for detecting estrogenic effects of exogenous chemicals. These homologous Vtg ELISAs will promote the use of guppy as a model organism to study estrogenic chemicals.

**Keywords** Environmental estrogens · Biomarker · Vitellogenin · Guppy · ELISA

## Introduction

Environmental estrogens, including both natural (estradiol, estrone, etc.) and synthesized estrogens (17 $\alpha$ -ethinylestradiol, bisphenol A, etc.), are widely present in aquatic environments and have aroused increasing concern for their adverse effects on the endocrine system of wildlife (Aoki et al. 2010; Jiang et al. 2012; Yan et al. 2012). Exposure to environmental estrogens

has been associated with abnormal gonad development, feminization of male individuals, and changes in sex ratios (Tyler and Jobling 2008; Jarque et al. 2015; Huang et al. 2016). To evaluate the estrogenic activity of chemical substances, several estrogen-sensitive proteins have been used as biomarkers including vitellogenin (Vtg), choriogenin, and zona radiata protein. Among them, Vtg induction in male and juvenile fish is the most recognized biomarker (Desforges et al. 2010; Gilannejad et al. 2016). Vtg concentrations in plasma or whole body homogenate (WBH) samples are normally measured by homologous enzyme-linked immunosorbent assay (ELISA), and the preparation of highly purified Vtg and its specific antibody are the key preconditions for a specific and sensitive ELISA (Peck et al. 2011; Wang et al. 2015). To date, homologous Vtg ELISAs have been established for a variety of fish species, such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), and fathead minnow (*Pimephales promelas*) (Brion et al. 2002; Fujiwara et al. 2005; Eidem et al. 2006). Compared with heterologous and hybrid ELISAs, homologous ELISA is more sensitive and robust, and thereby is considered

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to be the preferred immunoassay for accurate quantification of piscine Vtg (Fenske et al. 2001; Eidem et al. 2006).

Guppy (*Poecilia reticulata*), a small ovoviviparous fish of family Cyprinodontidae, have been intensively used as an experimental animal in toxicity studies for their numerous attractive features, including small size (males, 2.5–3.5 cm; females, 3.5–5.5 cm), easily cultured in the laboratory, growing quickly to sexual maturity (3 months), spawning occurs at any time of the year, and simply to separate females and males by their physical appearance (Fig. S1). Moreover, guppy was found to be susceptible to exogenous estrogens and thus was considered a promising model organism for investigating environmental estrogens (Cardinali et al. 2004; Volkova et al. 2012; Heintz et al. 2015). Previously, a homologous ELISA for guppy Vtg has been developed based on lipovitellin (Wang et al. 2017), but two guppy Vtgs were found in our following studies. Ohkubo et al. (2003) and Fujiwara et al. (2005) reported that different types of Vtgs in Japanese common goby (*Acanthogobius flavimanus*) and medaka had varied sensitivity to exogenous estrogens. However, Vtgs in guppy has not been purified, let alone the development of detection method for each Vtg. In this study, these guppy Vtgs were firstly purified, characterized, and used to prepare polyclonal antibodies, respectively. Then, two homologous sandwich ELISAs for quantification of guppy Vtgs were established. Furthermore, two ELISAs were used to measure Vtg induction in male guppy exposed to environmental concentrations of 17 $\alpha$ -ethinylestradiol (EE<sub>2</sub>) to assess their utility for detecting exogenous estrogens.

## Materials and methods

### Vtg induction and preparation of whole body homogenate

All animal research procedures were approved by the Institutional Animal Care and Use Committee of Ocean University of China, and the fish and rabbit were handled according to the National Institute of Health Guidelines for the handling and care of experimental animals. Three-month-old adult Red Albino guppies (length, 2.5 ± 0.2 cm; body weight, 0.37 ± 0.08 g) were obtained from a local dealer in Qingdao, China. In the laboratory, the fish were maintained in 50-L aquaria filled with 30-L dechlorinated tap water at 26 ± 1 °C, with 7.0 ± 0.1 mg/L dissolved oxygen and 14 h:10 h light-dark cycle.

Male guppies were induced to produce Vtg by exposure to 100 µg/L 17 $\beta$ -estradiol (E<sub>2</sub>, Sigma-Aldrich) in 5-L glass aquaria, and the exposure solution was renewed once daily. After 1 week, fish were anesthetized in 50 mg/L MS-222 (Sigma-Aldrich), weighed, and

homogenized in threefold volume of 10 mM PBS (pH 7.6). To avoid proteolysis of Vtg, 0.5 trypsin inhibiting units (TIU)/mL aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF) was added into PBS buffer. After centrifugation (10,000×g for 10 min at 4 °C), the supernatant was filtered through 0.45-µm filters and applied to the gel filtration column.

### Purification of guppy Vtgs

Vtgs in WBH of E<sub>2</sub>-exposed guppy were purified by a two-step chromatography process reported in the previous studies (Wang et al. 2015). In brief, 1 mL of WBH was loaded on a 70 × 1.6-cm Sephacryl S-300 HR column and then eluted with 25 mM Tris-HCl (pH 7.5) containing 0.07 M NaCl and 0.5 TIU/mL aprotinin. Putative Vtg fractions were collected and loaded on a 20 × 2.0-cm DEAE-Sepharose anion-exchange column. The loaded protein was eluted with a non-linear NaCl gradient (0.07, 0.10, 0.20, and 1.0 M NaCl in 25 mM Tris-HCl, pH 7.6), and among them, 1.0 M NaCl was used to regenerate the ion-exchange column. Fractions from eluted peaks were collected and analyzed by native-PAGE. Those fractions containing putative Vtg proteins were pooled and stored at –80 °C. Protein concentration was determined using the Bradford Assay, and all above purification steps were performed at 4 °C to reduce Vtg proteolysis.

### Characterization of guppy Vtgs

The molecular weight and purity of purified Vtgs were determined by gradient native-PAGE by the methods of Sun and Zhang (2001). The purified proteins (10 µL) were mixed with an equal volume of sample buffer and electrophoresed at 150 V at 4 °C for 120 min. The gels were stained with Coomassie Brilliant Blue R 250 (CBB R-250), and standard protein molecular weights (67–669 kDa, Amersham Biosciences, Uppsala, Sweden) were used to estimate the molecular weight of native Vtgs.

Purified proteins run in native-PAGE were stained separately with Schiff's reagent for carbohydrate, Sudan black B for lipid, and methyl green for phosphorous to confirm purified proteins as Vtgs as described by Jena et al. (2013).

The polypeptide subunits of purified Vtgs were determined by SDS-PAGE (4–9%) according to the method of Laemmli (1970). Samples were mixed with equal volume of sample buffer (25% glycerol, 0.1% bromophenol blue, 4% SDS, and 5%  $\beta$ -mercaptoethanol in 0.2 M Tris-HCl, pH 6.8), heated at 100 °C for 5 min, and then electrophoresed at 200 V for 90 min at room temperature. Molecular weight of polypeptide units was determined by comparing the mobility of molecular weight markers (Fermentas, Shenzheng, China).

Purified proteins were also identified by matrix-assisted laser desorption/ionization–time of flight/time of flight mass

spectrometry (MALDI-TOF/TOF MS) according to the method of Zhang et al. (2014). The protein bands were excised from native-PAGE gels and de-stained in 1 mL of CBB de-staining solution (25 mM ammonium bicarbonate and 50% acetonitrile (ACN)). The gel pieces were dehydrated successively in 50 and 100% ACN for 5 min before digestion in trypsin solution for 16 h at 37 °C. The supernatants were transferred to a new tube, and the resulting tryptic fragments were extracted with 67% ACN in 5% trifluoroacetic acid (TFA). The supernatants were pooled and vacuum dried. The dried peptides were mixed with a saturated solution of  $\alpha$ -cyano-4-hydroxy-trans-cinnamic acid in 50% ACN and 0.1% TFA and analyzed on an ABI5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, USA). Both the MS and MS/MS data were integrated and processed using the GPS 3.6 software (Applied Biosystems). Proteins were identified by searching the mass spectrometer data against the NCB-*All entries* database via MASCOT V2.3 tools (Matrix Science Ltd., London, UK).

### Production, purification, and label of anti-Vtg antibodies

Antisera against guppy Vtgs were raised in white rabbits by subcutaneous injection of 800  $\mu$ g purified proteins (1 mL) emulsified in complete Freund's adjuvant (Sigma) and boosted with the same dose in incomplete Freund's adjuvant (Sigma) for another four times at 2-week intervals. One week after the final injection, blood was collected and centrifugated to obtain the antisera. After determination of the titer by the indirect ELISA, the immunoglobulins (IgG) were purified from the antisera by affinity chromatography using a 1-mL HiTrap Protein G column (GE Healthcare). Purified anti-VtgAb and anti-VtgC IgG were labeled with horseradish peroxidase (HRP) using our previously described methods (Wang et al. 2015) after checking their purity by SDS-PAGE.

### Specificity of antisera to Vtgs

The WBH of control male and E<sub>2</sub>-exposed male guppy, and purified Vtgs separated by SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore) following the method of Towbin et al. (1979). The prepared antisera (anti-VtgAb and anti-VtgC, 1:2000 dilution) and HRP-conjugated goat anti-rabbit IgG (1:2000 dilution, Solarbio, China) were used as primary and secondary antibodies, respectively. Finally, the membranes were incubated in freshly prepared DAB substrate to visualize the bands.

### Development and validation of sandwich ELISA

Sandwich ELISAs were developed using a procedure modified from Mitsui et al. (2003). Microtiter plates (Costar, Cambridge, MA) were coated with 5  $\mu$ g/L anti-VtgAb or anti-VtgC IgG solution at 4 °C overnight. The wells were washed three times with 200  $\mu$ L of PBST (10 mM PBS containing 0.05% Tween-20) and blocked with 1% BSA-PBST. After three washes with PBST, 100  $\mu$ L of samples or standards (VtgAb and VtgC) serially diluted were added into each well and incubated at 37 °C for 1 h. The wells were washed five times and received 100  $\mu$ L/well of HRP-labeled anti-VtgAb or anti-VtgC IgG at serial dilutions (1:1250, 1:2500, 1:5000, 1:10,000). After another incubation and wash, the color was developed with 100  $\mu$ L of tetramethylbenzidine enzyme substrate (Solarbio, China). The reaction was stopped by adding 100  $\mu$ L of 2 M sulfuric acid, and absorbance values were measured at 450 nm by a microplate reader (Multiskan MK3, Thermo Scientific). Standards and samples were run in duplicate, and the performance of ELISA assays was evaluated by measuring their precision, sensitivity, and specificity according to the reported methods (Nilsen et al. 2004; Maltais et al. 2010). Moreover, the matrix effects of WBH in two ELISAs were evaluated by the methods described by Holbech et al. (2001).

To determine the cross reactivity of the two ELISA, two purified proteins were used as the standards and their reaction curves in two assays were compared.

### Vtg induction in male guppy exposed to EE<sub>2</sub>

Adult male guppies ( $n = 20$ ) were exposed to normal concentrations of 0, 2, 10, and 50 ng/L EE<sub>2</sub> (CAS-No.: 57-63-6, Sigma-Aldrich) in 10-L aquaria. The stock solution of EE<sub>2</sub> was prepared in dimethylsulfoxide (DMSO) and kept in light-proof glass reservoirs at 4 °C. The maximum concentration of DMSO in control and exposure solutions was below 0.001%. Each group of fish was exposed in two aquaria (ten fish per tank). The exposure solution was renewed once daily, and the fish were fed with brine shrimp twice daily. After 21 days of exposure, whole body, liver and caudal fin were collected, homogenated, and centrifugated as described above. Vtg concentrations in supernatant were quantified using two sandwich ELISAs directly.

### Statistical analysis

Vtg data are presented as the mean  $\pm$  standard deviation, and the differences between the control and exposed groups were assessed by one-way analysis of variance and Dunnett's test using SPSS 18.0 software (SPSS Inc., USA). Values were determined as significant when  $P < 0.01$ .

## Results

### Purification and characterization of guppy Vtgs

Three peaks were detected in WBH of E<sub>2</sub>-exposed guppy in gel filtration chromatography, and two protein bands were observed in the fractions of the first peak (Fig. S2). Fractions of this peak were pooled and subjected to exchange chromatography. When the gradient reached 0.15 M NaCl, a main peak was eluted followed by a small peak at 0.20 M NaCl (Fig. S3). Native-PAGE analysis showed that fractions of both peaks appeared as a single band. The purified proteins were stained positively with Sudan black B (Fig. 1a), Schiff's reagent (Fig. 1b), and methyl green (Fig. 1c).

In native-PAGE, apparent molecular weights of two purified proteins were estimated to be approximately 550 and 480 kDa, respectively (Fig. 2a). In SDS-PAGE, purified Vtg-550 generated three major bands with molecular weights of approximately 210, 126, and 102 kDa; Vtg-480 revealed a clear band of approximately 68 kDa (Fig. 2b).

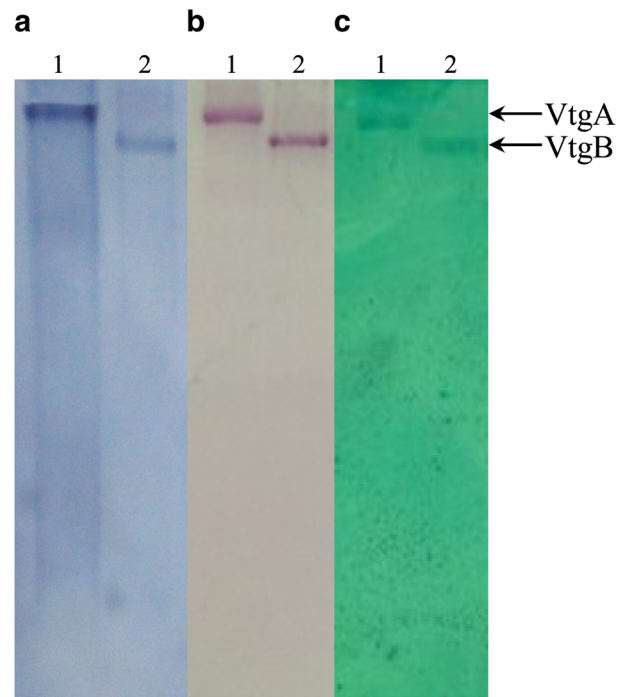
Protein bands in native-PAGE were identified by MALDI-TOF/TOF-MS (Fig. 3). The band of ~550 kDa had three peptide sequences: K.LLPGFGNSASNLEHR.V, K.YEGYLLGGLPEEGLAR.A, and K.YELGTEVLQTPIQLLR.V, which were highly homologous with guppy VtgAb (XP\_008406251.1, Fig. S4). MS/MS spectra of peptides from band of ~480 kDa (R.DPHGVQDVTLTIFLQK.H) showed a match with guppy VtgC sequence (XP\_008406324.1, Fig. S5). Blast results on the identified GenBank accession numbers revealed that Vtg-550 was actually VtgAb and Vtg-480 referred to as VtgC, a phosphovitinless Vtg (Fig. S6a, b). Moreover, the homology of amino acid sequences of the two Vtg isoforms was 19.65% (Fig. S7).

### Specificity of the antisera to guppy Vtgs

The titers of anti-VtgAb and anti-VtgC antisera were more than 1:128,000 and 1:64,000 by indirect ELISA (Tables S1 and S2). Western blot analysis showed that anti-VtgAb and anti-VtgC antisera reacted with purified VtgAb, VtgC, and WBH from E<sub>2</sub>-exposed male fish but did not react with the control male WBH (Fig. 4). Moreover, anti-VtgAb and anti-VtgC antisera detected different bands in WBH from E<sub>2</sub>-exposed male fish.

### Sandwich ELISAs

Sandwich ELISAs for VtgAb and VtgC were developed under the optimal conditions where the coating antibody diluted to 5 µg/mL, and HRP-labeled anti-VtgAb antibody and HRP-labeled anti-VtgC antibody were diluted 1:2500 and 1:1250, respectively (Fig. 5a, d). The working range of VtgAb ELISA was 7.8–1000 ng/mL ( $y = 1.157x - 0.839$ ,  $R^2 = 0.992$ ) with a



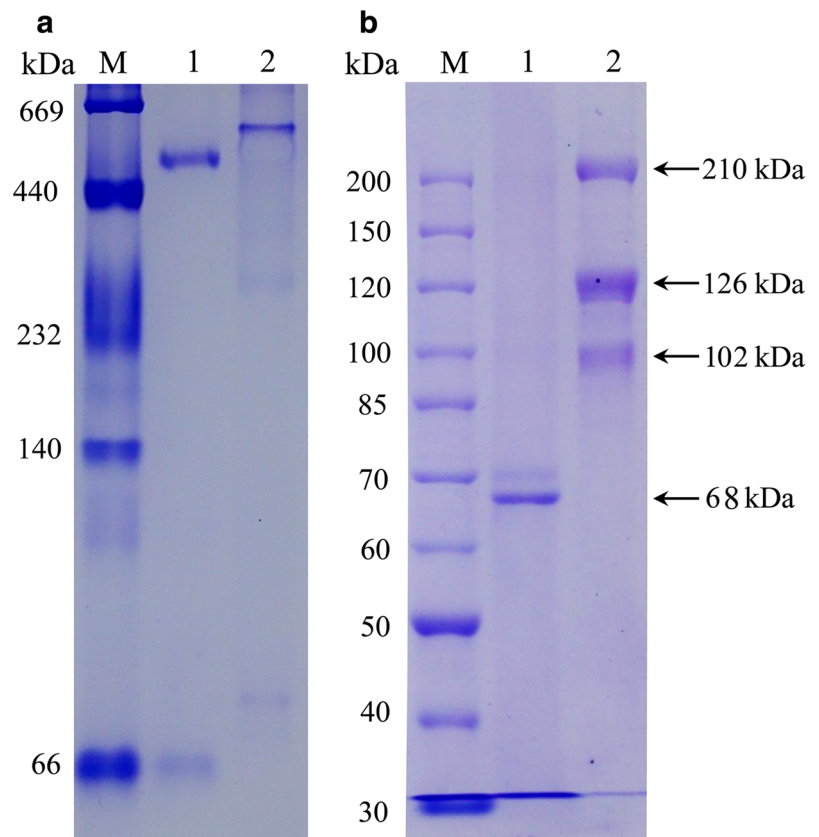
**Fig. 1** The purified proteins were run on a discontinuous 4–7.5% polyacrylamide gel and stained for lipid with Sudan black B (a), carbohydrates with Schiff's reagent (b), and phosphorous with methyl green (c). Lane 1, VtgAb; lane 2, VtgC

detection limit of 3.3 ng/mL (Fig. 5b, e). VtgC ELISA had a working range of 15.6–500 ng/mL ( $y = 1.248x - 0.967$ ,  $R^2 = 0.991$ ) and a detection limit of 9.5 ng/mL (Fig. 5e). Moreover, the dilution curves of WBH from E<sub>2</sub>-exposed guppy were parallel to Vtg standard curves, but no reaction was detected in control male WBH (Fig. 5c, f). The precision tests showed that intra- and inter-assay coefficients of variations for two sandwich ELISAs were both below 10% (Tables S3 and S4). Purified VtgAb and VtgC showed similar curves in the ELISA based on anti-VtgAb antibody, and the absorbance of VtgAb was higher than VtgC (Fig. 6a). In VtgC ELISA, the standard curve of VtgC was higher than that of VtgAb standard (Fig. 6b).

### Vtg induction in male guppy exposed to EE<sub>2</sub>

After a 21-day exposure to EE<sub>2</sub>, Vtg concentrations in male guppy were quantified by VtgAb and VtgC ELISAs, respectively (Fig. 7). Vtg concentrations in the control groups were below the detection limits, while exposure to 2 ng/L EE<sub>2</sub> significantly increased Vtg concentrations in the liver, fin, and whole body ( $P < 0.01$ ). Vtg concentrations increased with the exposure dose, and the maximum Vtg concentrations were found in liver of male guppy exposed to 50 ng/L EE<sub>2</sub>, with  $2.92 \pm 0.14$  and  $2.67 \pm 0.63$  µg/g measured by VtgAb and VtgC ELISAs, respectively.

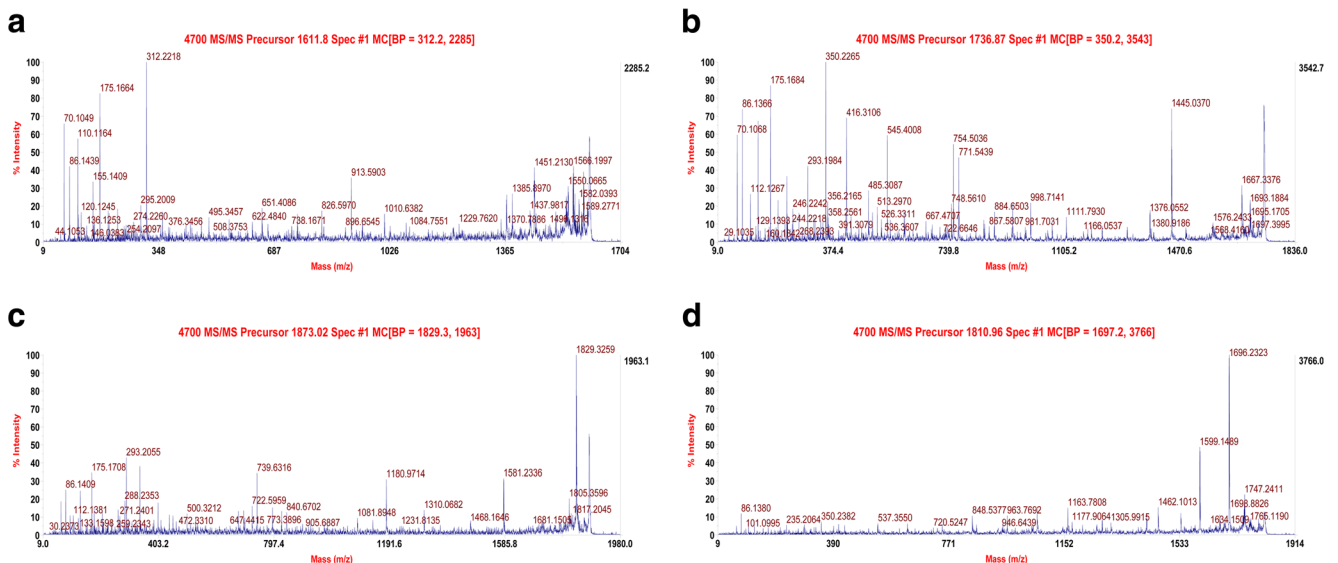
**Fig. 2** Native-PAGE (4–7.5%, **a**) and SDS-PAGE (4–9%, **b**) patterns of purified guppy VtgAb and VtgC. Gels were stained with Coomassie brilliant blue R250. M, protein marker; lane 1, purified VtgC; lane 2, purified VtgAb



## Discussion

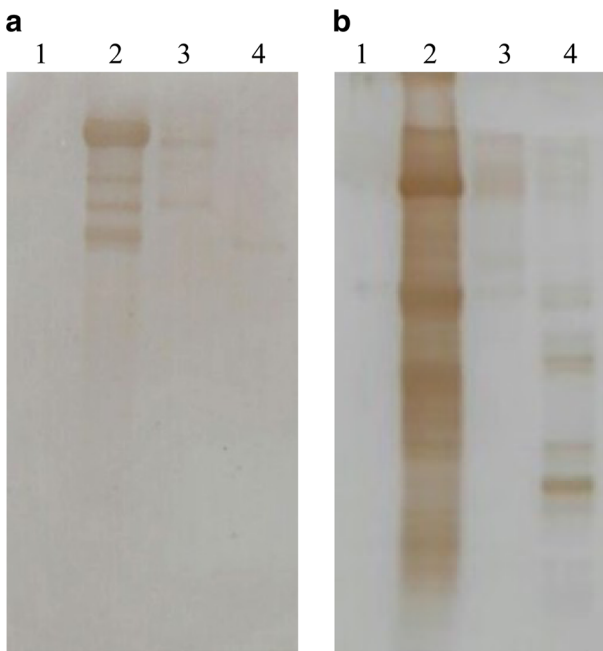
The present study developed two ELISAs for guppy Vtgs and validated their use for detecting estrogenic activity of chemical substance. Higher teleosts generally possess two complete Vtg

paralogues (VtgAa and VtgAb), which are composed of a signal peptide, a lipovitellin heavy chain (LvH), a phosvitin (Pv), a lipovitellin light chain (LvL), a  $\beta'$ -component and C-terminal coding region (Finn 2007). Many teleosts also possess an incomplete form of Vtg (VtgC), which is lacking a Pv domain



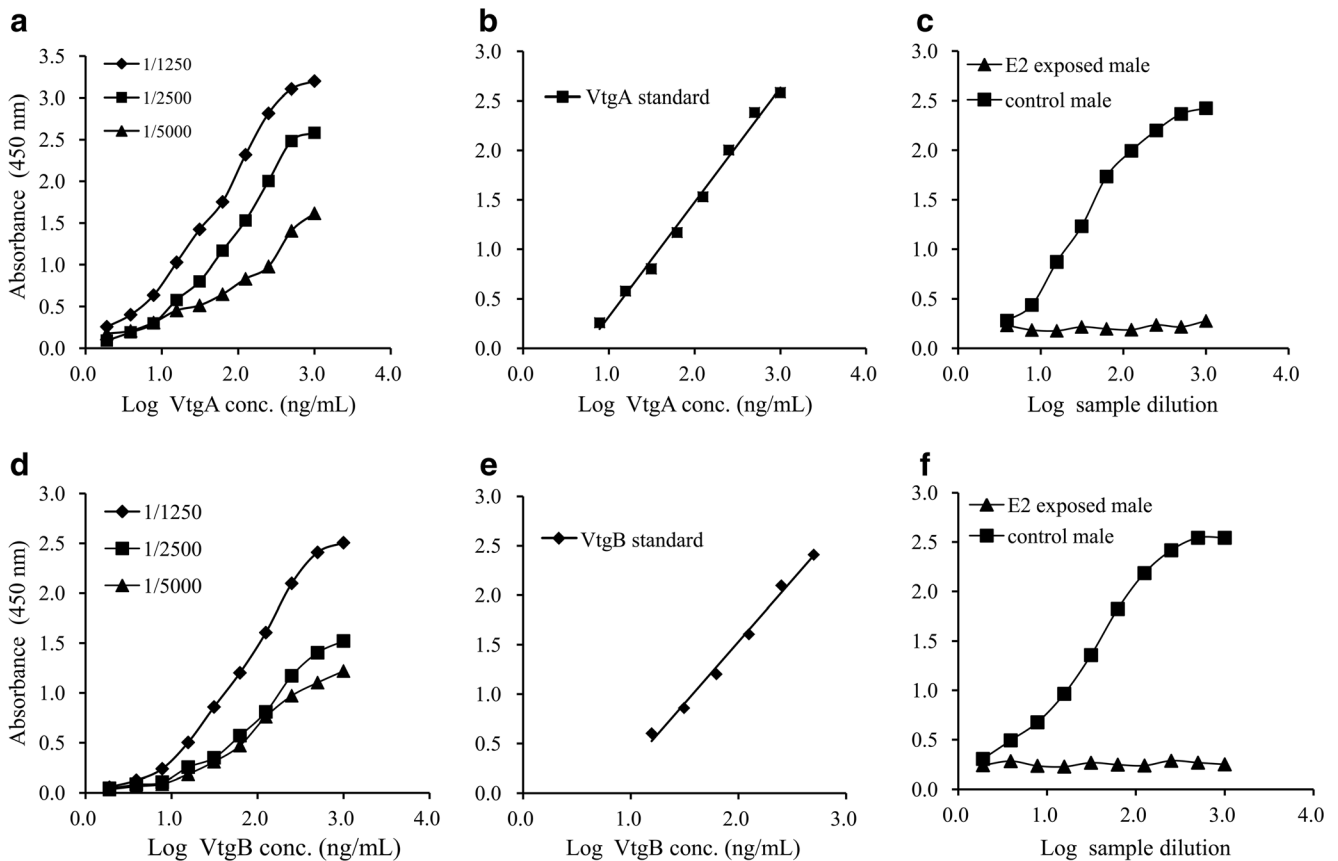
**Fig. 3** MS/MS spectra of K.LLPGFGNSASNLEHR.V (**a**), K.YEGLYLLGGLPEEGLAR.A (**b**), and K.YELGTEVLQTPIQLLR.V (**c**) from the purified protein with high molecular weight showed a match with VtgAb of *Poecilia reticulata* (XP\_008406251.1) in NCBI

database; MS/MS spectra of R.DPHGVQDVTLTIFLQK.H (**d**) from the purified protein with low molecular weight showed a match with VtgC of *Poecilia reticulata* (XP\_008406324.1) in NCBI database



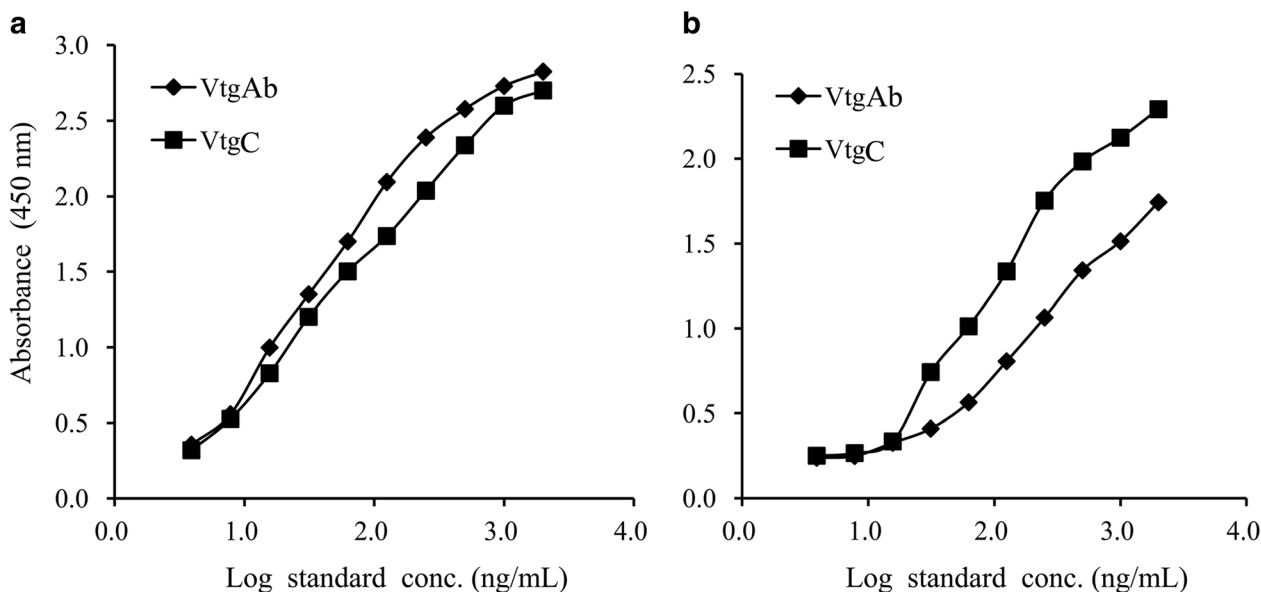
**Fig. 4** Western blot of purified VtgAb, VtgC, and whole body homogenate (WBH) using rabbit anti-VtgAb (a) and rabbit anti-VtgC polyclonal antiserum (b). Lane 1, WBH of control male guppy; lane 2, WBH of E<sub>2</sub>-exposed guppy; lane 3, purified VtgAb; lane 4, purified VtgC

(phosvitin-less Vtg) (Hiramatsu et al. 2006; Amano et al. 2010). It was reported that the characteristics of Vtgs in different oviparous fish vary considerably (Hiramatsu et al. 2002; Jeon et al. 2010). Ohkubo et al. (2003) found that Japanese common goby had two biochemically distinct Vtgs (VtgA, 530 kDa and VtgC, 320 kDa) with low cross-immunological reactivity. In barfin flounder (*Verasper moseri*), the molecular weights of two Vtgs were very similar to each other under native (VtgA, 530 kDa and VtgB, 550 kDa) and reducing conditions (Vtg A, 168 and VtgB, 175 kDa, Matsubara et al. 1999). Zebrafish was also found to possess at least two Vtg isoforms (520 and 440 kDa), but they were not purified separately (Brion et al. 2002). In this study, two proteins were successfully purified from WBH of E<sub>2</sub>-exposed guppy and their apparent molecular weights were approximately 550 and 480 kDa, similar to the results of other teleost Vtgs (Hiramatsu et al. 2006). The purified protein stained positively with methyl green, Sudan black B, and Schiff's reagent, indicating that both proteins were phospholipoglycoproteins, a common attribute of teleost Vtgs (Maltais and Roy 2009; Garnayak et al. 2013). In SDS-PAGE, two proteins were cleaved into different polypeptides from 68 to 210 kDa. Furthermore, MALDI-TOF/TOF-MS analysis showed that peptide sequences that matched were all in LvH



**Fig. 5** Determination of optimal dilution of HRP-labeled anti-VtgAb IgG (a) and anti-VtgC IgG (d), and a representative standard curve obtained for guppy VtgAb (b) and VtgC (e) and WBH dilution curves of male and

E<sub>2</sub>-induced male guppy in VtgAb sandwich ELISA (c) and VtgC sandwich ELISA (f)

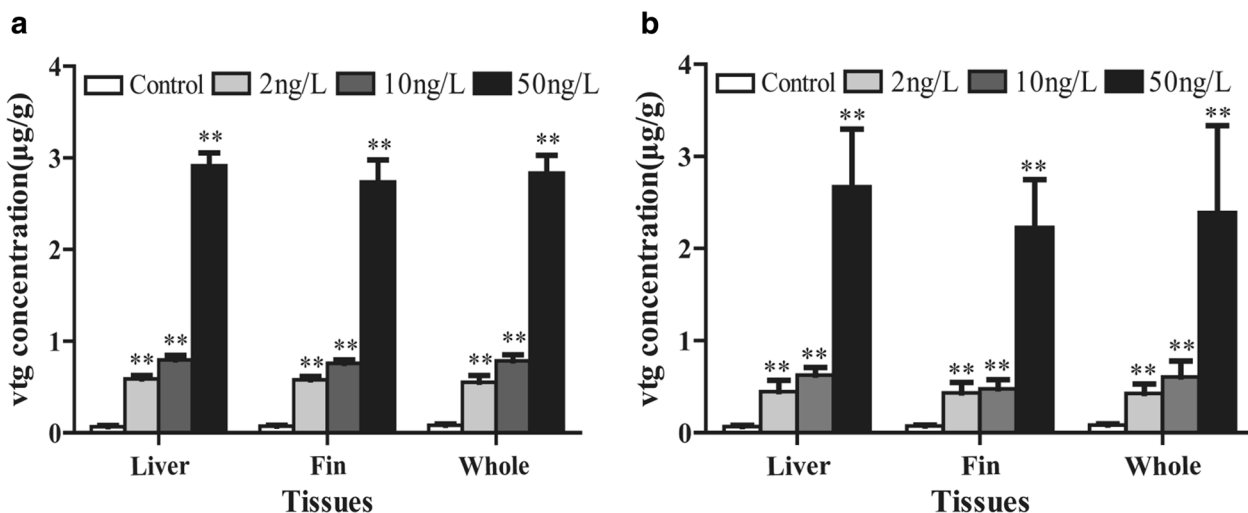


**Fig. 6** Cross reactivity of VtgAb ELISA (a) and VtgC ELISA (b). The purified VtgAb and Vtg C were used as the standards in two assays and their curves were compared

portion of the Vtgs, and they were highly homologous with VtgAb and VtgC, respectively. Based on above results, purified proteins were characterized as two isoforms of guppy Vtgs.

Using purified VtgAb and VtgC as antigens, two antisera with high titers were prepared. In Western blots, both anti-VtgAb and anti-VtgC reacted with purified VtgAb (VtgC), indicating that anti-VtgAb (anti-VtgC) showed cross-reactivity with VtgC (VtgAb) (Fujiwara et al. 2005; Maltais and Roy 2009). Two antisera reacted with WBH from E<sub>2</sub>-exposed male fish, while no visible band was observed in male WBH, confirming that two antisera were highly specific

to guppy Vtgs (Ohkubo et al. 2003; Wang et al. 2015). Based on purified proteins and their corresponding antibodies, two sandwich ELISAs were developed. ELISAs for VtgAb and VtgC have similar working range to Vtg ELISAs for other species, such as Chinese loach (*Misgurnus anguillicaudatus*, Shao et al. 2005) and Asian catfish (*Clarias batrachus*, Garnayak et al. 2013). VtgAb ELISA had a lower detection limit of 3.3 ng/mL, which was similar with guppy Vtg ELISA based on lipovitellin (3.1 ng/mL, Wang et al. 2017). While the detection limit of VtgC ELISA (9.5 ng/mL) was higher than the other two ELISAs. Parallelism between standard curves



**Fig. 7** Concentrations of Vtg in liver, tail fin, and whole body of adult male guppy ( $n = 20$ ) exposed to 2, 10, and 50 ng/L EE<sub>2</sub> for 21 days were measured by VtgAb ELISA (a) and VtgC ELISA (b). Values are means  $\pm$

SD and two asterisks indicate statistically significant difference from the control group (\*\* $P < 0.01$ )

and serial dilutions of WBH from E<sub>2</sub>-exposed guppy and no reaction of control male WBH in either ELISAs confirmed that two ELISAs could be used to quantify guppy Vtg (Roy et al. 2004; Peck et al. 2011). Moreover, the similar curves of VtgAb and VtgC in VtgAb ELISA revealed that VtgAb ELISA had strong cross reactivity to VtgC. While, VtgAb standards showed lower absorbance in VtgC ELISA, indicating that VtgC ELISA might underestimate VtgAb.

To validate the use of two ELISAs for detection of estrogenic activity of exogenous chemicals, EE<sub>2</sub> was chosen as a target substance to expose male guppy. EE<sub>2</sub> is one of the frequently detected xenoestrogens in aquatic environments with a concentration of 1–20 ng/L (Kolpin et al. 2002; Komori et al. 2004). The lowest observed effect concentration (LOEC) of Vtg induction in male zebrafish, medaka, and fathead minnows exposed to EE<sub>2</sub> were 1.67, 24.5 and 1.0 ng/L (Fenske et al. 2001; Japanese Ministry of Environment 2006; Pawlowski et al. 2004). In this study, significantly elevated Vtg concentration was found in male guppy exposed to 2 ng/L EE<sub>2</sub>, indicating that Vtg induction in guppy showed similar sensitivity to estrogenic chemicals as above species. Furthermore, Vtg induction was not only detected in WBH and liver but also in the caudal fin. Zhong et al. (2014) reported that Vtg could be detected in various extrahepatic tissues of EE<sub>2</sub>-exposed male zebrafish, including skin, eye, gonad, brain, and fin. Our study found that Vtg concentration in caudal fin was close to that in the WBH of male fish. Based on the above results, two Vtg ELISAs are proven to be capable of detecting estrogenic effect of exogenous chemicals. Considering the cross reactivity of two ELISAs, VtgAb ELISA should be prioritized for Vtg measurement to precisely quantify the Vtg concentration.

In conclusion, the present study characterized two forms of guppy Vtgs and developed two ELISAs for quantification of guppy Vtgs. Moreover, we validated the use of Vtg ELISAs for detecting estrogenic activities of EE<sub>2</sub> on male guppy at environmentally relevant concentrations. Therefore, two homologous Vtg ELISAs would provide useful tools for the use of guppy as a model organism to investigate environmental estrogens.

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