J Korean Soc Appl Biol Chem (2012) 55, 219–227
DOI 10.1007/s13765-012-1014-z

ORIGINAL ARTICLE

Identification, Purification, and Immunoassay of Stone Flounder (*Kareius bicolouratus*) Vitellogenin

Zongbao Pan · Hua Tian · Wei Wang · Jun Wang · Shaoguo Ru

Received: 27 October 2011 / Accepted: 3 January 2012 / Published Online: 30 April 2012

Abstract Vitellogenin (Vtg), the precursor of egg yolk proteins, serves as nutrients for the developing embryos. Vtg production in male fish has become an excellent biomarker for environmental estrogens. Thus, Vtg synthesis was induced by injection of 17β-estradiol (E_2) in stone flounder (*Kareius bicolouratus*). Plasma from uninduced and E_2-treated fish was subjected to native polyacrylamide gel electrophoresis (PAGE) and the gels were stained with Coomassie brilliant blue R250 for proteins, methyl green for phosphates, Sudan black B for lipids, and periodic acid-Schiff's reagent for carbohydrates. These analyses verified the presence of two phospholipoglycoproteins in the plasma from E_2-treated stone flounder, which were tentatively identified as Vtg. Vtg was then isolated by two purification procedures: gel filtration followed by anion exchange chromatography, or selective precipitation combined with anion exchange chromatography. The latter procedure was more effective at isolating Vtg with a high degree of purity. In native PAGE, the apparent molecular masses of Vtgs were determined to be 520 and 550 kDa, respectively. Two major bands with approximate molecular masses of 165 and 106 kDa together with two additional faint bands of lower molecular weights were observed after sodium dodecyl sulfate-PAGE under reducing conditions. These bands exhibited differential affinity for polyclonal antisera against red drum (*Sciaenops ocellatus*) Vtg in Western blot. The present study established the purification procedure and characterized the electrophoretic and immunological properties of stone flounder Vtg.

Keywords chromatography · selective precipitation · stone flounder · vitellogenin · Western blot

Z. Pan and H. Tian contributed equally.

Z. Pan · H. Tian · W. Wang · J. Wang · S. Ru (✉)
College of Marine Life Sciences, Ocean University of China, Qingdao 266003, China
E-mail: rusg@ouc.edu.cn

Introduction

Vitellogenin (Vtg), the precursor of egg yolk proteins in most oviparous vertebrates, is typically synthesized by hepatocytes in response to circulating estrogens, transported to the ovaries via the bloodstream, and incorporated into growing oocytes by receptor-mediated endocytosis (Wallace, 1985; Stifani et al., 1990; Specker and Sullivan, 1994; Hiramatsu, 2004). In the oocytes, Vtg is proteolytically cleaved into a characteristic suite of yolk proteins, including lipovitellin (Lv), phosvitin (Pv) and β'-component (β'), which serve as nutrients for the developing embryos (Specker and Sullivan, 1994; Hiramatsu et al., 2002b). Vtg normally occurs in sexually mature females, whereas male fish only synthesize minute amounts of Vtg, if any, due to the lack of endogenous estrogens (Copeland et al., 1986; Kishida and Specker, 1993; Koya et al., 1997; Susca et al., 2001). However, since males possess functional copies of *Vtg* gene, its expression can be readily induced by exposure to exogenous estrogens (Heppell et al., 1995). Therefore, abnormal production of Vtg, particularly in male fish, has become an excellent biomarker for environmental estrogens (Sumpter and Jobling, 1995; Tyler et al., 1999; Marin and Matozzo, 2004).

To date Vtgs have been purified and characterized in scores of fish species (Norberg, 1995; Matsubara et al., 1999; Roy et al., 2004; Sawaguchi et al., 2006). Teleost Vtgs are phospholipoglycoproteins with high molecular mass of 300–640 kDa, containing covalently linked carbohydrates and phosphates and non-covalently bound lipids, and thus can be identified in native gels stained with Coomassie brilliant blue (CBB) R250 for proteins, methyl green for phosphoproteins, Sudan black B for lipoproteins and periodic acid-Schiff's (PAS) reagent for glycoproteins (Chen et al., 1997). The application of these staining methods for characteristic groups has been proposed as a convenient technique for Vtg identification (Roubal et al., 1997; Magalhães et al., 2004). For qualitative or quantitative detection of Vtg, several methods have been developed, including radioimmunoassay (RIA), enzyme-linked immunosorbent

assay (ELISA), chemiluminescent immunoassay (CLIA), and Western blot (Norberg and Haux, 1988; Parks et al., 1999; Fukada et al., 2001; Roy et al., 2004; Meucci and Arukwe, 2005). Generally, these methods require a significant amount of purified Vtg for antibody production and for standards in sample determination. Since Vtgs from different species may show considerable heterogeneity after posttranslational modifications, the purification of Vtg for each species is a priority (Matsubara et al., 2003; Hiramatsu et al., 2006).

Various purification methods based on the high density, high surface charge or the size of Vtg have been established, such as density gradient ultracentrifugation, selective precipitation, anion exchange chromatography, and gel filtration chromatography (Komatsu wt al., 1996; Parks et al., 1999; Marx et al., 2001; Tolar et al., 2001). At present, the most frequently used procedure for Vtg purification is the two-step chromatographic method consisting of gel filtration followed by anion exchange chromatography or vice versa (Koya et al., 1997; Roy et al., 2004). In addition, several studies have demonstrated that it was advantageous to first precipitate plasma Vtg with Mg^{2+} and EDTA, and then further purify the dissolved Vtg by anion exchange chromatography (Norberg and Haux, 1988; Norberg, 1995). In the present study, these two procedures were both employed to establish the optimized conditions for stone flounder (*Kareius bicolouratus*) Vtg purification.

Vtg is usually measured by immunoassay and detected in Western blot using specific antibodies. Although considerable heterogeneity has been reported in the molecular mass, polypeptide, and chemical composition of Vtgs from different species (Mommsen and Walsh, 1988; Specker and Sullivan, 1994; Roubal et al., 1997), antibodies generally could recognize Vtg cross-reactive epitopes between related species (Nilsen, 1998). Moreover, several studies have shown Vtg cross-reactivity between unrelated species, even with monoclonal antibodies (Covens et al., 1987; Heppell et al., 1995). In our previous study, polyclonal antisera against purified red drum (*Sciaenops ocellatus*) Vtg had been raised in mice (Pan, 2011). Given that both stone flounder and red drum belong to Perciformes, purified stone flounder Vtg was included in Western blot to characterize its cross-reactivity with the antisera in the present study.

The stone flounder is an important commercial fish that inhabits inshore waters of China, the Korean Peninsula, and the Sakhalin Island. It belongs to the family of cold-water spawners with pelagic eggs, and the spawning season occurs in late autumn when water temperature falls below 10°C. The cultivating scale of this species grows rapidly in China, Japan, and South Korea. Under breeding conditions, human chorionic gonadotropin (hCG) and hormone-releasing hormone analogue (LRH-A) are extensively adopted to promote gonad development and ovulation. Yet, relatively little is known about the reproductive physiology of this species and no previous studies have examined its vitellogenesis. The present study describes the identification and purification of Vtg from plasma of E_2-treated stone flounder, and the fundamental properties of purified Vtg were characterized using electrophoretic and immunological assays.

Materials and methods

Fish and hormone treatment. Stone flounder with an average body weight of 100 g were purchased from a local commercial supplier and maintained in aquaria supplied with aerated, recirculated seawater (salinity 37‰) under natural photoperiod at ambient temperature (April-May). They were allowed a 5-day acclimation period. Each received one intramuscular injection of 17β-estradiol (E_2, Sigma-Aldrich, St Louis, MO) dissolved in ethanol and peanut oil (1:1, v/v) at a concentration of 10 mg/mL and the dose was 10 mg/kg body weight, whereas uninduced fish received injections of the vehicle only. Fish were starved during the 10-day experimental period.

Blood sampling and plasma preparation. After individuals were anesthetized by immersion in a tricane methane sulfonate solution (MS-222, 100 mg/L), blood was collected from the caudal vein with syringes prerinsed with phosphate-buffered saline solution containing 1600 international units (IU)/mL of sodium heparin and 200 trypsin inhibitor units (TIU)/mL of aprotinin (Sigma-Aldrich), and immediately transferred into 1.5-mL centrifuge tubes containing sodium heparin (25 IU), aprotinin (50 TIU), and phenylmethylsulfonyl fluoride (PMSF, 2 mM). Centrifugation was performed at 4000×g at 4°C for 5 min. Plasma was then collected and stored at –80°C. After blood collection, the animals were sacrificed and dissected to identify the gender and approximate reproductive status. Males and females could be differentiated by the color of the gonads, which were determined in stage II by morphological observation. The ovaries were pale yellow, whereas testes were milky white. Plasma from control female, control male, E_2-treated female, and E_2-treated male were each selected for the following experiments.

Purification of vitellogenin. Gel filtration was performed on a prepacked Sephacryl S-300 HR 16/70 column (GE Healthcare, Uppsala, Sweden). The column was equilibrated with 25 mM Tris-HCl and 0.07 M NaCl (pH 8.0). Plasma samples from E_2-treated and control males were applied separately onto the column, and eluted with the same buffer containing 200 TIU/L aprotinin at a flow rate of 1.0 mL/min at 4°C. The absorbance of the eluate was monitored at 280 nm. Fractions of 1.0 mL were collected and immediately stored at –80°C.

Selective precipitation was performed as described by Norberg (1995). Briefly, E_2-treated plasma was mixed with 0.02 M EDTA (pH 7.7) and 0.5 M $MgCl_2$ at 1.2:0.1. The Vtg fraction was precipitated by dilution of the mixture with ice-cold distilled water. The precipitate was separated by centrifugation (5000×g, 20 min) and resuspended in 0.5 mL of 1.0 M NaCl and reprecipitated with several volumes of distilled water. After centrifugation, the precipitate was redissolved in 0.5 mL of 1.0 M NaCl and diluted with 2.5 mL of 25 mM Tris-HCl (pH 8.0) that was added in small

portions. The resulting solution was applied to a DEAE-Sepharose column. Aprotinin (200 TIU) was added to the precipitate upon each removal of the resulting supernatant. All steps were performed at 4°C.

The Vtg solution from the Sephacryl S-300 column or selective precipitation was then fractionated on a DEAE-Sepharose FF 12/20 column (GE Healthcare) equilibrated with 25 mM Tris-HCl and 0.07 M NaCl (pH 8.0). Unbound proteins were eluted with the same buffer. Subsequently, the adsorbed proteins were eluted using a stepwise NaCl gradient of 0.10, 0.15, 0.18, 0.20, and 1.0 M (dissolved in 25 mM Tris-HCl, pH 8.0) at 1.0 mL/min. To reduce proteolysis, all chromatographic steps were conducted at 4°C, and all solutions used contained 200 TIU/L aprotinin. The elution profile was monitored at 280 nm. Fractions containing the desired protein peak were collected and immediately stored at –80°C. Estimates of molecular mass and purity were verified using native and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Gel electrophoresis. Discontinuous PAGE was carried out using an AE-6450 Dual Mini Slab Kit (Atto, Tokyo, Japan). The resolving gel contained 6.5% acrylamide for native PAGE or 10% for SDS-PAGE, whereas the stacking gel contained 4% of the same solution, according to Laemmli (1970). For native PAGE, samples were mixed with equal volumes of native sample buffer (0.20 M Tris-HCl, pH 6.8, 25% glycerol, 0.1% bromophenol blue), then electrophoresed at 150 V in Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) at 4°C. Five native PAGE markers of known molecular mass, thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and bovine serum albumin (67 kDa), were purchased from Amersham Biosciences (Uppsala, Sweden). For SDS-PAGE, samples were mixed with equal volumes of SDS sample buffer (0.16 M Tris-HCl, pH 6.8, 25% glycerol, 0.1% bromophenol blue, 4% SDS, 5% β-mercaptoethanol), then heated at 100°C for 3 min before loading into wells, and gels were run at 200 V at room temperature. PageRuler™ Unstained Protein Ladder for SDS-PAGE (Fermentas, Shenzhen, China), a mixture of 14 recombinants, highly purified proteins from 10 to 200 kDa, was used as molecular mass marker. The logarithm of the molecular mass markers was plotted against electrophoretic mobility, and the molecular mass of the proteins were calculated by linear regression.

Native gels were stained with 0.25% CBB R250 in 45% methanol and 10% acetic acid for 2 h and destained in 5% methanol and 7% acetic acid. Phosphoprotein was stained with methyl green as described by Cutting and Roth (1973). For lipoproteins, the gel was stained with Sudan black B (Prat et al., 1969). Glycoprotein was stained with PAS reagent according to the method of Zacharius et al. (1969). Diamine silver stains were applied to SDS gels (Wray et al., 1981).

Western blot. For Western blot analysis, proteins were electroblotted from the SDS-PAGE gel onto a polyvinylidene fluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA) using a DYCZ-40B transfer apparatus (Liuyi, Beijing, China). The transfer was done at 70 V for 5 h at 4°C using electrotransfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3). Non-specific binding sites on the membrane were blocked by immersion in blocking buffer (10 mM Tris, 0.15 M NaCl, 0.05% Tween 20, 5% skim milk, pH 7.5) at 4°C overnight. The membrane was incubated for 2 h with primary antibody (mouse anti-red drum Vtg) diluted 1:1000 in blocking buffer. Blots were then washed, incubated for 2 h with goat anti-mouse IgG horseradish peroxidase conjugate (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), diluted 1:2000 in blocking buffer, washed again, and placed into the DAB peroxidase substrate solution until bands developed. When bands became apparent, the membrane was removed and given a final rinse in ultrapure water (Super-Q™ water system, Millipore, Molsheim, France).

Results

Identification of vitellogenin. Initial experiments were conducted to identify Vtg in the blood of stone flounder. Plasmas from uninduced and E_2-treated fish were subjected to native PAGE (Fig. 1A). E_2 treatment of both sexes resulted in the appearance of two major high-molecular-mass proteins with low electrophoretic mobility, which were absent from that of uninduced fish. According to the staining intensity, it seemed that synthesis of these two proteins increased dramatically at the expense of other plasma proteins after E_2 injection. Besides CBB R250 staining, the two bands stained positively with methyl green for phosphates (Fig. 1B), Sudan black B for lipids (Fig. 1C) and PAS reagent for carbohydrates (Fig. 1D), indicating that these two proteins were both phospholipoglycoproteins. Based on these results, the two E_2-inducible proteins were tentatively identified as stone flounder Vtg.

Purification of vitellogenin. The elution profiles of uninduced male and E_2-treated male plasmas on Sephacryl S-300 column are shown in Fig. 2. Through comparison of their respective chromatogram, it could be seen that E_2-treatment induced a dominant UV-absorbing peak that had an elution volume between 80 and 95 mL following the void volume (V_0) peak, whereas no peak was observed at the corresponding position for the control plasma. By native PAGE, besides the E_2-induced 520 and 550 kDa proteins, which were designated as Vtg-520 and Vtg-550, another 230 kDa band was present in obtained fractions (Fig. 3, lane 3). Consequently, fractions of the main peak (elution volume between 84 and 92 mL) were further subjected to anion exchange chromatography (Fig. 4). Following three small peaks eluted by stepwise NaCl gradient of 0.07, 0.10, and 0.15 M, the main component was eluted when the gradient reached 0.18 M NaCl. However, native PAGE analysis showed Vtg-520 experienced a serious loss during the DEAE-Sepharose chromatography step, whereas the staining intensity of the 230 kDa band was increased (Fig. 3, lane 4), suggesting it might be a proteolytic product.

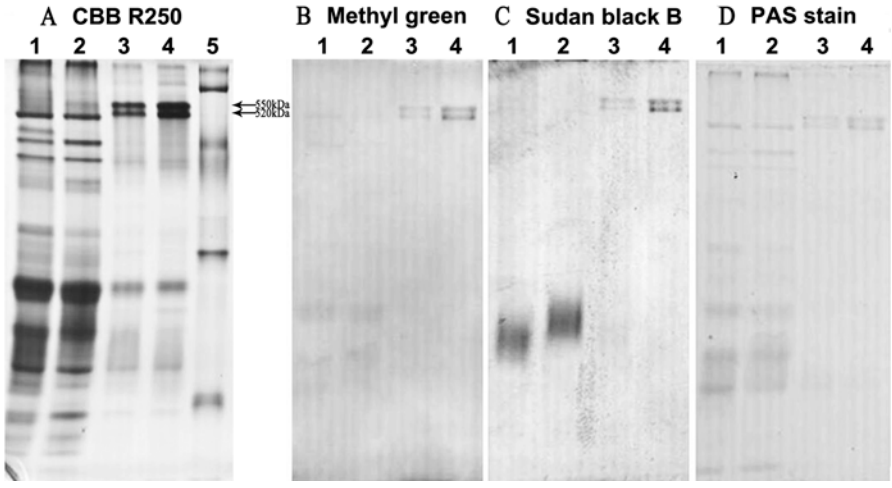

Fig. 1 Electrophoretic separation of plasma proteins from stone flounder. Plasma was subjected to native PAGE and stained using CBB R250 (A), methyl green (B), Sudan black B (C), and PAS reagent (D). Lane 1, plasma from control female; lane 2, plasma from control male; lane 3, plasma from E_2-treated female; lane 4, plasma from E_2-treated male; lane 5, HMW native marker.

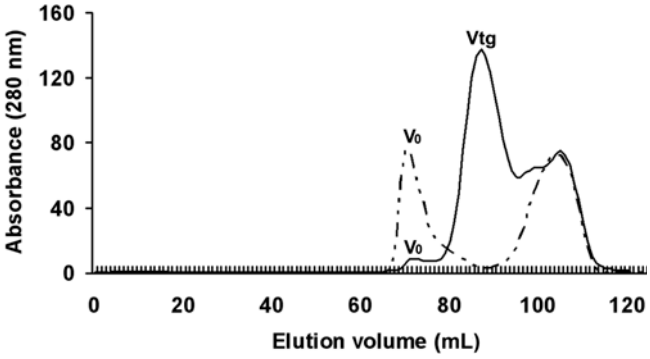

Fig. 2 Elution profiles of stone flounder proteins in plasmas from uninduced male and E_2-treated male on a Sephacryl S-300 column. Fractions 84–92, representing Vtg, were collected for further analysis. Dashed line: plasma from uninduced male. Solid line: Plasma from E_2-treated male. V_0: void volume.

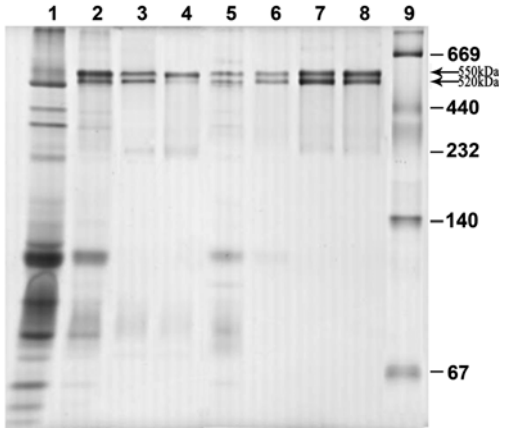

Fig. 3 Native PAGE of plasma and fractions of stone flounder. Lane 1, plasma from control male; lane 2, plasma from E_2-treated male; lane 3, Vtg fractions from Sephacryl S-300 chromatography; lane 4, Vtg fractions from DEAE-Sepharose chromatography following Sephacryl S-300 chromatography; lane 5, proteins precipitated by $MgCl_2$-EDTA (single precipitation); lane 6, proteins precipitated by $MgCl_2$-EDTA and distilled water (double precipitation); lanes 7-8, Vtg fractions from DEAE-Sepharose chromatography of proteins obtained by double precipitation; lane 9, HMW native marker. The gels were stained with CBB R250.

Selective precipitation of Vtg from E_2-treated plasma was performed by first mixing the plasma with EDTA and $MgCl_2$, which was not sufficient to precipitate the Vtg fraction. When the mixture was diluted with several volumes of distilled water, a precipitate was formed. Analysis on native PAGE revealed that this precipitate consisted largely of Vtg but was still contaminated by some other plasma proteins (Fig. 3, lane 5). Reprecipitation with distilled water removed the contaminating proteins to a large extent (Fig. 3, lane 6). Further purification of the second precipitate was performed using DEAE-Sepharose medium. Only a few components were eluted at the lower NaCl concentrations of the stepwise gradient before the Vtg peak elution at 0.18 M NaCl (data not shown). When analyzed by native PAGE, the collected fractions consisted of only two major bands with approximately the same staining intensity, representing the native Vtg-520 and Vtg-550 (Fig. 3, lanes 7–8).

When analyzed by SDS-PAGE, the Vtgs purified by the latter procedure (selective precipitation combined with anion exchange chromatography) resolved into two major bands corresponding to 165 and 106 kDa, a faint band of 92 kDa and a barely detectable band of 73 kDa (Fig. 5A, lanes 3–4). The fractions obtained by the former procedure (gel filtration followed by anion exchange chromatography) that eluted at the later positions in the peak showed an identical electrophoretic pattern (Fig. 5A, lane 2), whereas there were a few more faint bands for those eluted earlier (Fig. 5A, lane 1).

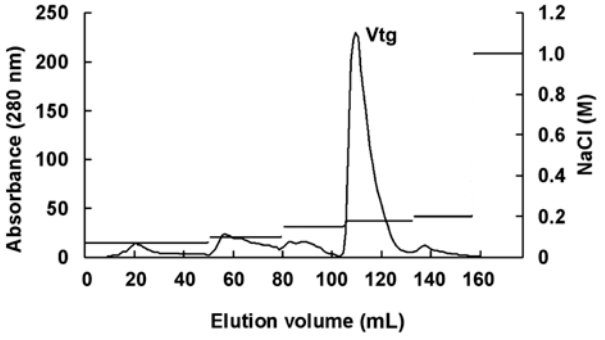

Fig. 4 DEAE-Sepharose column chromatographic purification of Vtg fractions from the Sephacryl S-300 chromatography (Fractions 84–92 in Fig. 2). The bars indicate NaCl gradients for each elution volume, 0.07, 0.10, 0.15, 0.18, 0.20, and 1.0 M.

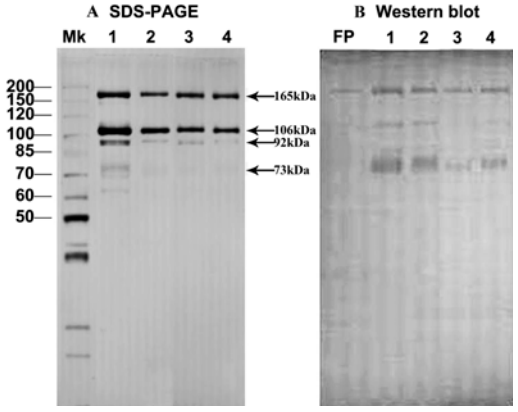

Fig. 5 SDS-PAGE (A) and Western blot (B) of purified fractions from stone flounder. Lanes 1-2, Vtg fractions from DEAE-Sepharose chromatography following Sephacryl S-300 chromatography; Lanes 3-4, Vtg fractions from DEAE-Sepharose chromatography of proteins obtained by double precipitation. Mk: HMW SDS marker. FP: plasma form control female.

Western blot. To further characterize the nature of Vtg, Western blot analysis was performed after SDS-PAGE under reducing conditions. All four bands showed positive reactions to the polyclonal antisera against red drum Vtg, though differential affinity was observed (Fig. 5B). Although the staining intensity was almost identical in SDS-PAGE gels, the 165 kDa band was prominent in Western blot, whereas the 106 kDa band was merely a faint band. This lack of a cross-reaction was especially true for purified Vtg obtained by selective precipitation combined with anion exchange chromatography (Fig. 5B, lanes 3–4). Only a rather weak cross-reaction was observed for the 92 kDa band. Interestingly, the barely detectable 73 kDa band in SDS gel was even more apparent than the 165 kDa band in Western blot, indicating its high affinity for the heterogeneous antisera. Although no Vtg band was detected in native PAGE, a faint band (165 kDa) was observable by Western blot in the plasma from a control female (Fig. 5B, lane FP).

Discussion

The present study describes the identification, purification, and partial characterization of stone flounder Vtg. Vitellogenesis has been induced by injection of E_2 in scores of marine teleosts, such as Atlantic halibut (*Hippoglossus hippoglossus*), barfin flounder (*Verasper moseri*), smooth flounder (*Pleuronectes putnami*), and red seabream (*Pagrus major*) (Norberg, 1995; Matsubara et al., 1999; Roy et al., 2004; Sawaguchi et al., 2006). In the present study, E_2-treatment resulted in the production of two high-molecular-mass proteins in stone flounder, which were not present in the control plasma. According to the positive staining with methyl green, Sudan black, and PAS reagent for phosphates, lipids and carbohydrates, respectively, both proteins were verified as phospholipoglycoproteins. Thus, they were tentatively identified as two forms of Vtg in stone flounder. E_2 injection generally evokes a significant increase in total plasma proteins as compared with control fish, with most of the elevated total proteins being attributable to Vtg (Komatsu et al., 1996; Roubal et al., 1997). Judging by the staining intensity of plasma proteins in native gels, a dramatic elevation was observed for stone flounder Vtg in E_2-treated plasma, whereas the synthesis of other plasma proteins was suppressed, which was also the case with Atlantic halibut (Norberg, 1995). Therefore, the relatively high concentration of Vtg in E_2-treated plasma makes it an ideal material for Vtg purification.

Various methods have been developed for the purification of Vtg with high purity and minimal breakdown, with the most frequently used being the two-step chromatographic method (Roubal et al., 1997; Roy et al., 2004). Selective precipitation followed by anion exchange chromatography was also used to isolate Vtg of brown trout and Atlantic halibut with high efficacy (Norberg and Haux, 1988; Norberg, 1995). Both methods were employed in the present study to establish the optimized procedure for isolation of stone flounder Vtg. When purified by the two-step chromatographic method, the 230 kDa band observed in fractions from gel filtration was probably derived from native Vtg, because it was not present in E_2-treated plasma and the staining intensity increased after further purification with anion exchange chromatography. Moreover, despite the addition of the serine protease inhibitor aprotinin in the elution buffer (200 TIU/L), Vtg-520 was still affected by proteolysis. Although successful for purification of Vtg from English sole, eelpout (*Zoarces viviparus*), and smooth flounder (Roubal et al., 1997; Korsgaard and Pedersen, 1998; Roy et al., 2004), the present study showed the two-step chromatography was not effective for stone flounder Vtg purification. Alternatively, anion exchange chromatography following selective precipitation was performed as in the case of Atlantic halibut (Norberg, 1995).

It was originally suggested that teleost Vtgs might not be precipitated with $MgCl_2$ and EDTA by the method of Wiley et al. (1979), because they are less phosphorylated than Vtgs from other

vertebrates (Selman and Wallace, 1983; Mommsen and Walsh, 1988; Tyler and Sumpter, 1990). Vtg of mummichog (*Fundulus heteroclitus*) and copper redhorse (*Moxostoma hubbsi*) failed to be precipitated using this method (Kanungo et al., 1990; Maltais and Roy, 2009), yet Vtgs from several other species, such as turbot (*Scophthalmus maximus*), sole (*Solea vulgaris*) and Atlantic halibut have been precipitated (Nunez-Rodriguez et al., 1989; Silversand and Haux, 1989; Norberg, 1995), in most cases by slight modifications of the ratio of EDTA : $MgCl_2$. Vtg may not precipitate when its plasma level is low. In the rainbow trout, the concentration had to be higher than 7–8 mg/mL before the precipitate was formed (Norberg and Haux, 1985). Factors that influence the precipitation of Vtg from aqueous solutions also include the molecular mass and polarity of the protein, the pH of the solution, and the binding sites at the protein surface (Maltais and Roy, 2009). In the present study, precipitation of stone flounder Vtg occurred not by mixing plasma with EDTA and $MgCl_2$, but by dilution of the mixture with a relatively large volume of distilled water, because Vtg gradually and easily precipitates in solution with low ionic strength (Lee et al., 1997). Reprecipitation removed the majority of co-purifying plasma proteins from Vtg. It followed that selective precipitation was more feasible and effective in comparison with gel filtration chromatography, and further purification on DEAE-Sepharose column completely removed other impurity proteins. Because the solution of the reprecipitate was immediately applied onto the DEAE-Sepharose column, degradation of Vtg-520 was presumed to be closely related to the freeze-thaw cycle during two-step chromatography. Thus, selective precipitation combined with anion exchange chromatography is more appropriate for the isolation of Vtg from other plasma proteins in stone flounder.

In the present study, there were two E_2-inducible phosphoglycoproteins that were verified as stone flounder Vtg. Up until the mid-1990s, however, only a single type of Vtg had been identified in teleosts, including brown trout (*Salmo trutta*), turbot, sole, and striped bass (*Morone saxatilis*) (Norberg and Haux, 1985; Nunez-Rodriguez et al., 1989; Silversand and Haux, 1989; Kishida et al., 1992). Recent investigations have confirmed the presence of multiple forms of Vtg in a range of species, such as barfin flounder, white prech (*Morone Americana*), Japanese common goby, and red seabream (Matsubara et al., 1999; Hiramatsu et al, 2002a, 2002b; Matsubara et al., 2003; Ohkubo et al., 2003; Hiramatsu et al, 2006; Sawaguchi et al., 2006; Finn, 2007b). Based on their primary structures and/or known physiological functions, these various Vtgs are classified into three groups, including two types of "complete" Vtg (VtgA and VtgB) with the domains arranged in linear fashion as NH_2- heavy chain lipovitellin (LvH)- phosvitin (Pv)- light chain lipovitellin (LvL)- β'-component (β')- C-terminal coding region (CT)- COOH, and a smaller, "incomplete" Vtg lacking a Pv domain (VtgC) (Hiramatsu et al., 2002a, 2005). It appears that members of higher teleost taxa (Paracanthopterygii and Acanthopterygii) generally express both VtgA and VtgB, and the Pv-less Vtg seems to be widely present among teleosts (Wang et al., 2000; Matsubara et al., 2003; Hiramatsu et al., 2006). The apparent molecular masses of stone flounder Vtgs were calculated to be 520 and 550 kDa, which are in accordance with those obtained in winter flounder (*Pseudopleuronectes americanus*) (500 kDa), turbot (530 kDa), smooth flounder (520 kDa), and in particular barfin flounder (500–520 and 530–550 kDa) (Silversand and Haux, 1989; Matsubara et al., 1999; Roy et al., 2004). Vtg-520 and Vtg-550 were eluted as a single peak during chromatography, probably because there was no significant difference in their molecular masses, and may reflect the possibility that the two forms of Vtg share similarity in surface charge to a certain extent. Although it is possible that Vtg-520 is a proteolytic variant of Vtg-550 or just a charge isomer of Vtg, as discussed in murrel (*Channa punctatus*) (Sehgal and Goswami, 2005), it should be noted that stone flounder belongs to the marine spawners with pelagic eggs that are supposed to synthesize at the least both complete types of Vtg (Matsubara et al., 1999; Sawaguchi et al., 2006). Furthermore, the result of the present study was comparable to those of barfin flounder (Matsubara et al., 1999). Therefore, it could be speculated that Vtg-520 and Vtg-550 represent the two distinct forms of Vtg in stone flounder.

When subjected to SDS-PAGE, the copurified Vtgs were resolved into two major bands (Vtg-165 and Vtg-106) and two faint bands (Vtg-92 and VTg-73). Vtg-165 was supposed to be an intact Vtg monomer, as its molecular mass approximates those of other flatfish, such as turbot (185 kDa), Atlantic halibut (160 kDa), winter flounder (175 kDa), and barfin flounder (168 and 175 kDa) (Silversand and Haux, 1989; Norberg, 1995; Hartling et al., 1997; Matsubara et al., 1999). Vtg-106 was presumed to represent the LvH domain, as is the case with barfin flounder (107 kDa for LvHA and 94 kDa for LvHB), Japanese common goby (110 kDa), and red seabream (110 kDa for LvH) (Matsubara et al., 1999; Ohkubo et al., 2003; Sawaguchi et al., 2006). The occurrence of proteolytic degradation of Vtg during purification process has been reported in scores of species (Norberg, 1995; Roubal et al., 1997; Roy et al., 2004; Maltais and Roy, 2009). Take common goby for example, its monomer Vtg-178 was almost completely degraded into Vtg-110 after purification (Ohkubo et al., 2003). Though a fully conserved LvL cleavage site (LvL-CS) has been confirmed in barfin flounder and red seabream (Matsubara and Sawano, 1995; Sawaguchi et al., 2006), cleavages of Vtg monomers could have occurred at different sites. For instance, in mosquitofish, despite having the LvL-CS, the VtgAa form remains as conjugates that range from full Vtg to Pv-LvL, whereas in the VtgAb form, only LvH was identified (Sawaguchi et al., 2005). According to the predicted molecular masses of Vtg monomer and conjugates of various fish species (Finn, 2007b), taking the high susceptibility of Vtg-520 to degradation into account, Vtg-165 is possibly the monomer of Vtg-550, and Vtg-106 may represent the LvH domain of Vtg-520. As for Vtg-92 and Vtg-73, they could well have been derived from Vtg-165 from the point of view of molecular mass. Therefore, proteolytic

degradation of Vtg appeared inevitable in spite of serine protease inhibitors and low temperature.

There are many factors that can influence the stability of Vtgs. Inadequate laboratory conditions such as performing the chromatography at room temperature, would result in a dramatic decline in stability of the Vtg during preparation (Silversand and Haux, 1989; Norberg, 1995). Repeated freeze-thaw cycles of the same plasma samples also easily degrade Vtg (Norberg and Haux, 1988; Silversand et al., 1993). In addition, Inaba et al. (1997) characterized a trypsin-like serine protease, which degrades Vtg in the plasma of tilapia (*Oreochromis niloticus*); hence serine protease inhibitors are generally included during blood collection and purification in order to prevent proteolysis. Even though the various isolation steps were carefully carried out to minimize proteolysis by performing purification at low temperature, minimizing repeated freeze/thawing steps, and addition of protease inhibitor, our results suggested that stone flounder Vtgs, in particular Vtg-520, are susceptible to proteolysis. This is obviously observed in SDS-PAGE, though Vtgs appeared to be 'intact' when analyzed by native PAGE. Therefore, the degradation of Vtg could be due to "proteolytic nicking", that is, although no significant change was observed in the purified Vtg on its intact molecular mass, yet the subunit structure was altered, as revealed by SDS-PAGE under denaturing and reducing conditions (Hiramatsu et al., 2006; Sawaguchi et al., 2006). Similar proteolysis was also observed in other species, such as common goby (Vtg-530 versus Vtg-110 instead of Vtg-178) (Ohkubo et al. 2003) and mosquitofish (Vtg-400 versus Vtg-112 instead of Vtg-142) (Sawaguchi et al., 2005).

Moreover, it was suggested that Vtg from cold-water spawners, such as halibut and cod, in which the molecule could be temperature-sensitive, may be more susceptible to the degradation at elevated temperatures than Vtg of warm-water species (Norberg, 1995). Recent studies verified the disparate involvement of VtgA and VtgB and their derivative yolk proteins in regulation of oocyte hydration (Matsubara et al., 1999; Hiramatsu et al., 2002a; Matsubara et al., 2003; Hiramatsu et al., 2006; Sawaguchi et al., 2006), and researches on barfin flounder, haddock and Atlantic halibut exhibited that different Vtgs experience distinct extent of proteolysis (Matsubara et al., 1999; Reith et al., 2001; Finn, 2007a). This, in return, could reflect the difference in stability of VtgA and VtgB to a certain extent. This may be the case with Vtg-520, since stone flounder is a cold-water marine species that spawns pelagic eggs when water temperature falls below 10ºC.

Antigenic determinants are indispensable for antigen-antibody recognition, and can be classified into two categories including sequential epitope and conformational epitope. In Western blot using SDS gels, sequential epitope is recognized by antibodies via its linear sequence of around five to six amino acids. Vtg is known to be an ancient protein which has further developed during evolution. However, some homologous sequences have been conserved, and they may very well be the sequential epitopes recognized by Vtg antibodies (Byrne et al., 1989; Marx et al., 2001). In Western blot, all stone flounder Vtg bands were recognized by the antisera against red drum Vtg, yet exhibited different affinities. Vtg-165 was well recognized, only a weak cross-reaction was observed for Vtg-92, and though being a faint band in SDS-PAGE, Vtg-73 was more apparent in Western blot. The high affinity of Vtg-73 for the antisera could be related to proteolysis, just as in the case of brown trout (*Slamo trutta*), in which extensive degradation of Vtg and hence a possible increase in the number of immunoreactive sites yielded apparent Vtg levels much higher than the real ones (Norberg and Haux, 1988). Therefore, it is likely that the cross-reactive epitopes are mainly located in the Pv-LvL-β'c conjugate (Vtg-73) rather than in the LvH domain (Vtg-92). As for Vtg-106, supposed to be the LvH domain of Vtg-520, showed rather weak affinity for the antisera, and selective precipitation followed by anion exchange chromatography further lowered its cross-reactivity to some extent.

The same Western blot also revealed a faint 165 kDa band in plasma from a control female. This band likely represents minute amount of Vtg, although no Vtg band was detected in native PAGE. In the wild, stone flounder begins its spawning season in autumn when water temperature falls below 10ºC. Our experiments were conducted from April to May, under natural photoperiod at ambient temperature. At the end of the experiments, the fish were dissected to determine the sex and reproductive status, and the gonads were in stage II by morphological observation. Hence, this control female may have been in the early stage of vitellogenesis, when the morphological changes associated with gonadal maturation were not obvious to the naked eye.In conclusion, the present study established the purification method for Vtg and partially characterized the electrophoretic and immunological properties of Vtg from stone flounder.

Acknowledgment This research was supported by the National Natural Science Foundation of China (30870250).

References

Byrne BM, Gruber M, and Ab G (1989) The evolution of egg yolk proteins. *Prog Biophys Molec Biol* **53**, 33–69.

Chen JS, Sappington TW, and Raikhel AS (1997) Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. *J Mol Evol* **44**, 440–51.

Copeland PA, Sumpter JP, Walker TK, and Croft M (1986) Vitellogenin levels in male and female rainbow trout (*Salmo gairdneri* Richardson) at various stages of the reproductive cycle. *Comp Biochem Physiol B Biochem Mol Biol* **83**, 487–93.

Covens M, Covens L, Ollevier F, and Loof AD (1987) A comparative study of some properties of vitellogenin (Vg) and yolk proteins in a number of freshwater and marine teleost fishes. *Comp Biochem Physiol B Biochem Mol Biol* **88**, 75–80.

Cutting JA and Roth TF (1973) Staining of phospho-proteins on acrylamide gel electropherograms. *Anal Biochem* **54**, 386–94.

Finn RN (2007a) The maturational disassembly and differential proteolysis of paralogous vitellogenins in a marine pelagophil teleost: A conserved mechanism of oocyte hydration. *Biol Reprod* **76**, 936–48.

Finn RN (2007b) Vertebrate yolk complexes and the functional implications of phosvitins and other subdomains in vitellogenins. *Biol Reprod* **76**, 926–35.

Fukada H, Haga A, Fujita T, Hiramatsu N, Sullivan CV, and Hara A (2001) Development and validation of chemiluminescent immunoassay for vitellogenin in five salmonid species. *Comp Biochem Physiol A Mol Integr Physiol* **130**, 163–70.

Hartling RC, Pereira JJ, and Kunkel JG (1997) Characterization of a heat-stable fraction of lipovitellin and development of an immunoassay for vitellogenin and yolk protein in winter flounder (*Pleuronectes americanus*). *J Exp Zool* **278**, 156–66.

Heppell SA, Denslow ND, Folmar LC, and Sullivan CV (1995) Universal assay of vitellogenin as a biomarker for environmental estrogens. *Environ Health Perspect* **103**, 9–15.

Hiramatsu N, Chapman RW, Lindzey JK, Haynes MR, and Sullivan CV (2004) Molecular characterization and expression of vitellogenin receptor from white perch (*Morone americana*). *Biol Reprod* **70**, 1720–30.

Hiramatsu N, Cheek AO, Sullivan CV, Matsubara T, and Hara A (2005) Vitellogenesis and endocrine disruption. In *Biochemistry and Molecular Biology of Fishes*, Mommsen TP, and Moon T, pp. 431–71, Elsevier Science Press, Amsterdam, Netherlands.

Hiramatsu N, Matsubara T, Fujita T, Sullivan CV, and Hara A (2006) Multiple piscine vitellogenins: Biomarkers of fish exposure to estrogenic endocrine disruptors in aquatic environments. *Mar Biol* **149**, 35–47.

Hiramatsu N, Matsubara T, Hara A, Donato DM, Hiramatsu K, Denslow ND et al. (2002a) Identification, purification and classification of multiple forms of vitellogenin from white perch (*Morone americana*). *Fish Physiol Biochem* **26**, 355–70.

Hiramatsu N, Matsubara T, Weber GM, Sullivan CV, and Hara A (2002b) Vitellogenesis in aquatic animals. *Fish Sci* **68**, 694–9.

Inaba K, Buerano CC, Natividad FF, and Morisawa M (1997) Degradation of vitellogenins by 170 kDa trypsin-like protease in the plasma of the tilapia, *Oreochromis niloticus*. *Comp Biochem Physiol B Biochem Mol Biol* **118**, 85–90.

Kanungo J, Petrino TR, and Wallace RA (1990) Oogenesis in *Fundulus heteroclitus*. VI. Establishment and verification of conditions for vitellogenin incorporation by oocytes in vitro. *J Exp Zool* **254**, 313–21.

Kishida M, Anderson TR, and Specker JL (1992) Induction by á-estradiol of vitellogenin in striped bass (*Morone saxatilis*): Characterization and quantification in plasma and mucus. *Gen Comp Endocrinol* **88**, 29–39.

Kishida M and Specker JL (1993) Vitellogenin in tilapia (*Oreochromis mossambicus*): induction of two forms by estradiol, quantification in plasma and characterization in oocyte extract. *Fish Physiol Biochem* **12**, 171–82.

Komatsu M, Matsunoto W, and Hayashi S (1996) Protease activity appeared after trypsin treatment of the purified vitellogenin from eel *Anguilla japonica*. *Comp Biochem Physiol B Biochem Mol Biol* **113**, 561–71.

Korsgaard B and Pedersen KL (1998) Vitellogenin in *Zoarces viviparus*: purification, quantification by ELISA and induction by estradiol-17á and 4-nonylphenol. *Comp Biochem Physiol C Toxicol Pharmacol* **120**, 159–66.

Koya Y, Matsubara T, Ikeuchi T, Adachi S, and Yamauchi K (1997) Annual changes in serum vitellogenin concentrations in viviparous eelpout, *Zoarces elongatus*. *Comp Biochem Physiol A Mol Integr Physiol* **118**, 1217–23.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **277**, 680–7.

Lee FY, Shih TW, and Chang CF (1997) Isolation and characterization of the female-specific protein (vitellogenin) in mature female hemolymph of the freshwater prawn, *Macrobrachium rosenbergii*: Comparison with ovarian vitellin. *Gen Comp Endocrinol* **108**, 406–15.

Magalhães I, Ledrich ML, Pihan JC, and Falla J (2004) One-step, non-denaturing purification method of carp (*Cyprinus carpio*) vitellogenin. *J Chromatogr B Analyt Technol Biomed Life Sci* **799**, 87–93.

Maltais D and Roy RL (2009) Purification and partial characterization of vitellogenin from shorthead redhorse (*Moxostoma macrolepidotum*) and copper redhorse (*Moxostoma hubbsi*) and detection in plasma and mucus with a heterologous antibody. *Fish Physiol Biochem* **35**, 241–54.

Marin GM and Matozzo V (2004) Vitellogenin induction as a biomarker of exposure to estorogenic compounds in aquatic environment. *Mar Pollut Bull* **48**, 835–9.

Marx A, Sherry J, Hassen PD, and Hock B (2001) A new monoclonal antibody against vitellogenin from rainbow trout (*Oncorhynchus mykiss*). *Chemosphere* **44**, 393–9.

Matsubara T, Nagae M, Ohkubo N, Andoh T, Sawaguchi S, Hiramatsu N et al. (2003) Multiple vitellogenins and their unique roles in marine teleosts. *Fish Physiol Biochem* **28**, 295–9.

Matsubara T, Ohkubo N, Andoh T, Sullivan CV, and Hara A (1999) Two forms of vitellogenin, yielding two distinct lipovitellins, play different roles during oocyte maturation and early development of barfin flounder, *Verasper moseri*, a marine teleost that spawns pelagic eggs. *Dev Biol* **213**, 18–32.

Matsubara T and Sawano K (1995) Proteolytic cleavage of vitellogenin and yolk proteins during vitellogenin uptake and oocyte maturation in barfin flounder (*Verasper moseri*). *J Exp Zool* **272**, 34–45.

Meucci V and Arukwe A (2005) Detection of vitellogenin and zona radiata protein expressions in surface mucus of immature juvenile Atlantic salmon (*Salmo salar*) exposed to waterborne nonylphenol. *Aquatic Toxicol* **73**, 1–10.

Mommsen TP and Walsh PJ (1988) Vitellogenesis and oocyte assembly. In *Fish Physiology*, Hoar WS, and Randall DJ, pp. 347–406, Academic Press, New York, NY.

Nilsen BM, Berg K, Arukwe A, and Goksøyr A (1998) Monoclonal and polyclonal antibodies against fish vitellogenin for use in pollution monitoring. *Mar Environ Res* **46**, 153–7.

Norberg B (1995) Atlantic halibut (*Hippoglossus hippoglossus*) vitellogenin: induction, isolation and partial characterization. *Fish Physiol Biochem* **14**, 1–13.

Norberg B and Haux C (1988) An homologous radioimmunoassay for brown trout (*Salmo trutta*) vitellogenin. *Fish Physiol Biochem* **5**, 59–68.

Norberg B and Haux C (1985) Induction, isolation and a characterization of the lipid content of plasma vitellogenin from two Salmo species: Rainbow trout (*Salmo gairdneri*) and sea trout (*Salmo trutta*). *Comp Biochem Physiol B Biochem Mol Biol* **81**, 869–76.

Nunez-Rodriguez J, Kah O, Geffard M, and Le Menn F (1989) Enzyme linked immunosorbent assay (ELISA) for sole (*Solea vulgaris*) vitellogenin. *Comp Biochem Physiol B Biochem Mol Biol* **92**, 741–6.

Ohkubo N, Mochida K, Adachi S, Hara A, Hotta K, Nakamura Y et al. (2003) Development of enzyme-linked immunosorbent assays for two forms of vitellogenin in Japanese common goby (*Acanthogobius flavimanus*). *Gen Comp Endocriol* **131**, 353–64.

Pan ZB (2011) Preparation of polyclonal antisera against vitellogenin from *Sciaenops ocellatus* and its application to screening pesticides with estrogenic activities. MS Thesis, Ocean University of China, Qingdao, China.

Parks LG, Cheek AO, Denslow ND, Heppell SA, McLachlan JA, LeBlanc GA et al. (1999) Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. *Comp Biochem Physiol C Toxicol Pharmacol* **123**, 113–25.

Prat JP, Lamy JN, and Weill JD (1969) Coloration des lipoprotéines après électrophorèse en gel de polyacrylamide. *Bull Soc Chim Biol* **51**, 1367.

Reith M, Munholland J, Kelly J, and Finn RN (2001) Lipovitellins derived from two forms of vitellogenin are differentially processed during oocyte maturation in haddock (*Melanogrammus aeglefinus*). *J Exp Zool* **291**, 58–67.

Roubal WT, Lomax DP, Willis ML, and Johnson LL (1997) Purification and partial characterization of English sole (*Pleuronectes vetulus*) vitellogenin. *Comp Biochem Physiol B Biochem Mol Biol* **118**, 613–22.

Roy RL, Morin Y, Courtenay SC, and Robichaud P (2004) Purification of vitellogenin from smooth flounder (*Pleuronectes putnami*) and

measurement in plasma by homologous ELISA. *Comp Biochem Physiol B Biochem Mol Biol* **139**, 235–44.

Sawaguchi S, Kagawa H, Ohkubo N, Hiramatsu N, Sullivan CV, and Matsubara T (2006) Molecular characterization of three forms of vitellogenin and their yolk protein products during oocyte growth and maturation in red seabream (*Pagrus major*), a marine teleost spawning pelagic eggs. *Mol Reprod Dev* **73**, 719–36.

Sawaguchi S, Koya Y, Yoshizaki N, Ohkubo N, Andoh T, Hiramatsu N et al. (2005) Multiple vitellogenins (Vgs) in mosquitofish (*Gambusia affinis*): identification and characterization of three functional Vg genes and their circulating and yolk protein products. *Biol Reprod* **72**, 1045–60.

Sehgal N and Goswami SV (2005) Vitellogenin exists as charge isomers in the Indian freshwater murrel, *Channa punctatus* (Bloch). *Gen Comp Endocrinol* **141**, 12–21.

Selman K and Wallace RA (1983) Oogenesis in *Fundulus heteroclitus*. III. Vitellogenesis. *J Exp Zool* **226**, 441–57.

Silversand C and Haux C (1989) Isolation of turbot (*Scophthalmus maximus*) vitellogenin by high performance chromatography. *J Chromatogr* A **478**, 387–97.

Silversand C, Hyllner SJ, and Haux C (1993) Isolation, immunochemical detection and observations of the instability of vitellogenin from four teleosts. *J Exp Zool* **267**, 587–97.

Specker JL and Sullivan CV (1994) Vitellogenesis in fishes: status and perspectives. In *Perspectives in Comparative Endocrinology*, Davey KG, Peter RE, and Tobe SS, pp. 304–15, National Research Council, Ottawa, Canada.

Stifani S, Le Menn F, Rodriguez JN, and Schneider WJ (1990) Regulation of oogenesis: the piscine receptor for vitellogenin. *Biochem Biophys Acta* **3**, 271–9.

Sumpter JP and Jobling S (1995) Vitellogenesis as a biomarker for estrogenic contaminants of the aquatic environment. *Environ Health Perspect*. **103** (Suppl 7), 173–8.

Susca V, Corriero A, Bridges CR, and de Metrio G (2001) Study of the sexual maturity of female bluefin tuna: Purification and partial characterization of vitellogenin and its use in an enzyme-linked immunosorbent assay. *J Fish Biol* **58**, 815–31.

Tolar JF, Mehollin AR, Watson RD, and Angus RA (2001) Mosquitofish (*Gambusia affinis*) vitellogenin: Identification, purification, and immunoassay. *Comp Biochem Physiol C Toxicol Pharmacol* **128**, 237–45.

Tyler CR and Sumpter JP (1990) The purification and partial characterization of carp, Cyprinus carpio, vitellogenin. *Fish Physiol Biochem* **8**, 111–20.

Tyler CR, van Aerle R, Hutchinson TH, Maddix S, and Trip H (1999) An in vivo testing system for endocrine disruptors in fish early life stages using induction of vitellogenin. *Environ Toxicol Chemistry* **18**, 337–47.

Wallace RA (1985) Vitellogenesis and oocyte growth in nonmammalian vertebrates. In *Oogenesis*, Browder LW, pp. 127–77, Plenum Press, New York, NY.

Wang H, Yan T, Tan JTT, and Gong Z (2000) A zebrafish vitellogenin gene (*Vg3*) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene. *Gene* **256**, 303–10.

Wiley HS, Opresko L, and Wallace RA (1979) New methods for the purification of vertrebrate vitellogenin. *Analyt Biochem* **97**, 145–52.

Wray W, Bonlikas T, Wray VP, and Hancock R (1981) Silver staining of proteins in polyacrylamide gels. *Anal Biochem* **118**, 197–203.

Zacharius RM, Zell IL, Merrison JH, and Nordlock JJ (1969) Glycoprotein staining following electrophoresis on acrylamide gels. *Anal Biochem* **30**, 148–52.