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Development of a lipovitellin-based sandwich ELISA for quantification of vitellogenin in surface mucus and plasma of goldfish (*Carassius auratus*)

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ABSTRACT

Goldfish (*Carassius auratus*) vitellogenin (Vtg) is an efficient biomarker for estrogen contamination in aquatic environments. In this study, Vtg and lipovitellin (Lv) were purified from the plasma of 17β-estradiol (E_2)-induced male goldfish and unfertilized eggs of females, and were used to generate polyclonal antibodies against Vtg (anti-Vtg) and Lv (anti-Lv), respectively. SDS-PAGE and Western blot were performed to confirm the specificity of the two antibodies and the immunological similarity between Vtg and Lv. As anti-Lv recognized more antigen epitopes than anti-Vtg, it was used to develop a sandwich enzyme-linked immunosorbent assay (ELISA) for goldfish Vtg with purified Lv as the standard. The detection limit of the assay was 1.82 ng/mL, and the working range was 3.9–250 ng/mL. The use of Lv instead of Vtg as the standard provided greater precision and strengthened the robustness of the sandwich ELISA. Western blot and the Lv-based ELISA were used to detect Vtg inductions in surface mucus and plasma of E_2-induced goldfish. The surface mucus Vtg level in E_2-induced males was significantly higher than that in the control males and E_2-induced females, and was much closer to the plasma Vtg level in E_2-induced males than that in E_2-induced females. Therefore, the surface mucus Vtg level of male goldfish may be a reliable indicator of estrogenic activity in the aquatic environment.

1. Introduction

Many substances that mimic endogenous estrogens, termed "environmental estrogens," have the potential to alter normal gonad development, sex ratios, and even the sizes of wildlife populations (Allen et al., 1999; Jobling and Tyler, 2003; Sumpter et al., 2005). To evaluate the estrogenic activity of chemicals, the Organization for Economic Co-operation and Development (OECD) and the U.S. Environmental Protection Agency (US EPA) have recommended a fish screening assay protocol that uses vitellogenin (Vtg) as a core endpoint (EPA, 2006; OECD, 2006a, 2006b, 2010). Goldfish (*Carassius auratus*), a cyprinid with a worldwide distribution, has a moderate body size that permits blood and organ sampling and is highly tolerant to pollution; therefore, Goldfish is widely used in the detection of estrogens in the aquatic environment (Deguchi et al., 2008; Ishibashi et al., 2001; Yan et al., 2012). An enzyme-linked immunosorbent assay (ELISA) for quantifying goldfish Vtg has been developed using purified Vtg, while goldfish Vtg was found to be easily degraded during storage at −80 °C (Wang et al., 2015). The breakdown products of Vtg are more immunogenic than Vtg itself, which would poses a challenge for the use of Vtg as a standard in quantitative assays (Arukwe and Goksøyr, 2003; Nilsen et al., 2004). For example, while using purified English sole (*Pleuronectes vetulus*) Vtg that showed some degradation after 3–4 months in storage at −80 °C as a standard, Lomax et al. (1998) observed a change in the slope of the ELISA standard curve. Thus, the degradation of goldfish Vtg may affect the precise quantification of Vtg. Hennies et al. (2003) proposed to seek a stable Vtg fragment to serve as the standard for immunoassays. Vtg is a complex phospholipoglycoprotein synthesized in the liver of sexually mature females, secreted into the blood, and then transported to the ovary, where it is rapidly cleaved into lipovitellin (Lv), phosvitin (Pv), and β'-component (β'-c) (Hiramatsu et al., 2002; Yamane et al., 2013). The immunogenicity of Lv is similar to that of Vtg (Amano et al., 2008). Hartling et al. (1997) also found that winter flounder (*Pleuronectes americanus*) Lv was a heat-stable protein, showing no degradation after being heated at 85 °C for 7 min. Thus, Lv may be a good substitute for Vtg in the ELISA assay.

Plasma Vtg is one of the most common endpoints for estrogenic activity evaluation (Brodeur et al., 2006; Deguchi et al.,

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2008; Tian et al., 2009). To measure Vtg, blood is collected from the caudal vein using a syringe, which is an invasive procedure for the fish. Recently, Vtg in surface mucus was proposed as an alternative for the detection of plasma Vtg because mucus collection is relatively easy and non-invasive (Maltais and Roy, 2007; Moncaut et al., 2003; Van Veld et al., 2005). The feasibility of mucus Vtg has been tested in many species, including Atlantic salmon (*Salmo salar*), copper redhorse (*Moxostoma hubbsi*), and shorthead redhorse (*Moxostoma macrolepidotum*), by evaluating the estrogenic activity of nonylphenol and β-estradiol 3-benzoate (Maltais et al., 2010; Meucci and Arukwe, 2005). Although goldfish is a good model species for evaluating the estrogenic activity of chemicals, the detection of mucus Vtg in this species has not been reported.

In the present study, a highly robust lipovitellin-based sandwich ELISA for goldfish Vtg was developed and validated. The ELISA was used to measure Vtg levels in surface mucus and plasma of E_2-induced goldfish. The results confirmed that surface mucus Vtg in male goldfish was an alternative endpoint for estrogenic compound screening and provided a non-invasive method to detect estrogenic contamination in aquatic environments.

2. Materials and methods

2.1. Experimental fish

Goldfish (9.4 ± 0.7 cm in length; 21.6 ± 4.6 g in weight) were purchased from a local supplier and maintained in 70-L aquaria filled with 50 L of dechlorinated tap water (temperature: 21 ± 2 °C) under a 14/10-h light/dark cycle. The fish were fed with a pelletized diet (Minjiang Aquarium Industry Co., Ltd., China) once daily. After a 2-week acclimation period in the laboratory, the fish were used for the experiments. Fish were handled according to the National Institute of Health Guidelines for Handling and Care of Experimental Animals. The animal utilization protocol was approved by the Institutional Animal Care and Use Committee of the Ocean University of China.

2.2. Vitellogenin induction and preparation of egg extracts

The fish were induced to produce Vtg by receiving one intraperitoneal injection of 17β-estradiol (E_2, Sigma, St. Louis, MO, USA) at a dose of 10 mg/kg body weight. Control fish received one injection of solvent vehicle (ethanol) alone. After 7 days, goldfish were anesthetized with 60 mg/L MS-222 (Sigma). Blood was withdrawn from the caudal vein using chilled heparinized syringes. To prevent the proteolytic breakdown of Vtg, 3 mM phenylmethylsulfonyl fluoride (PMSF) was added to centrifuge tubes. Plasma was obtained by centrifugation (5000*g*, 8 min) and stored at −80 °C.

Unfertilized eggs (5 g) were removed from the ovaries of vitellogenic females and homogenized using a glass homogenizer with 25 mL of 20 mM Tris–HCl (pH 7.5) containing 100 mM NaCl, 10 mM EDTA, and 1 mM PMSF. The egg homogenates were centrifuged at 8000*g* for 20 min at 4 °C. The supernatant was filtered through a 0.45-μm filter and stored at −80 °C.

2.3. Purification of Vtg and Lv

Vtg and Lv were purified by a two-step purification method (Wang et al., 2015). Briefly, 1 mL of plasma from E_2-induced male goldfish and egg extracts were applied separately onto a Sephacryl S-300 HR 16/70 column (GE Healthcare, Uppsala, Sweden) and eluted with 25 mM Tris–HCl (pH 7.5) containing 0.07 M NaCl. Eluted fractions of the first peak containing protein were collected, pooled, and then fractionated on a DEAE-Sepharose FF 12/20 column (GE Healthcare). Elution of the bound proteins was performed using a non-linear NaCl gradient (0.1, 0.2, and 1.0 M in 25 mM Tris–HCl, pH 7.5), and the eluted peak of 0.2 M NaCl was collected. All purification steps were performed at 4 °C with buffers containing 1 mM PMSF to reduce degradation. Purified Vtg and Lv were stored at −80 °C. Protein concentrations of purified Vtg and Lv were determined by the Bradford assay, using bovine serum albumin as the standard.

2.4. Gel electrophoresis and staining

The purified proteins were analyzed using native polyacrylamide gel electrophoresis (PAGE) (4–7.5%). First, 5-μL samples were mixed with equal volumes of sample buffer (0.20 M Tris–HCl, pH 6.8, 25% glycerol, 0.1% bromophenol blue) and then electrophoresed at 150 V in Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3) at 4 °C. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R 250 (CBB R-250), Schiff reagent (Fairbanks et al., 1971), methyl green (Cutting and Roth, 1973), and Sudan black B (Prat et al., 1969), respectively.

Sodium dodecyl sulfate (SDS)-PAGE was used to analyze 5 μL of plasma from control and E_2-induced male goldfish, purified Vtg, and purified Lv according to the methods described by Laemmli (1970). The loaded amount of purified Vtg and purified Lv was approximately 9 μg. Samples were mixed with equal volumes of SDS sample buffer (0.16 M Tris–HCl, pH 6.8, 25% glycerol, 1% bromophenol blue, 4% SDS, 5% β-mercaptoethanol), heated for 5 min at 100 °C, and then electrophoresed at 200 V at room temperature. After electrophoresis, the gels were stained with CBB R-250, and the molecular weights of Vtg and Lv were estimated using an unstained protein ladder (20–200 kDa; Thermo Scientific, Lithuania).

2.5. Antibody production

Polyclonal antisera against purified Vtg or Lv were raised in rabbits (~3 kg in weight) using standard immunological methods. Briefly, each rabbit was immunized subcutaneously with a primary dose of 0.5 mL of purified Vtg/Lv (800 μg) emulsified with an equal volume of Freund's complete adjuvant. Booster doses (600 μg of purified proteins emulsified with an equal volume of Freund's incomplete adjuvant) were administered at 2-week intervals. The antisera were checked by the immunodiffusion technique before immunization. When the antibody titer was sufficient, blood was withdrawn and allowed to clot overnight at 4 °C. The antisera were collected by centrifuging at 3000*g* for 10 min at 4 °C. Immunoglobulin G (IgG) was fractionated from the antisera by affinity chromatography on a HiTrap Protein G column (GE Healthcare) according to the manufacturer's instructions. The purified IgG fraction was condensed by 50% saturated ammonium sulfate and then dialyzed against 0.01 M phosphate-buffered saline (PBS, pH 7.4) overnight at 4 °C. The purified IgG fraction was stored at −80 °C, and the protein concentration of purified IgG was determined by the Bradford method.

2.6. Western blot

Western blot was conducted according to the method of Towbin et al. (1979). Proteins separated by SDS-PAGE were electroblotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore). After transfer, the membranes were blocked with 5% skim milk in TBST (100 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.5) overnight at 4 °C. Next, the membranes were incubated with anti-Vtg or anti-Lv as the primary antiserum at a dilution of 1:1000 in TBST for 4 h at room temperature. After three washes,