J. ENVIRON. TOXICOL.
Vol. 19, No. 4, 353~357 (2004)

Development of Quantitative Vitellogenin ELISAs for Goldfish (*Carassius auratus*) used in Endocrine Disruptor Screening

Chun-Ri Li, Kwang-Tae Kim, Andre Kim, Kyu-Hyuck Chung[1], Dong-Kyoo Kim[2], Shin-Won Kang and Jang-Su Park[※]

Department of Chemistry and Centre for Innovative Bio-Physio Sensor Technology, Pusan National University, Busan 609-735, Korea
[1]College of pharmacy, Sungkyunkwan University, 440-746, Korea
[2]Department of chemistry and biohealth product research centre, Inje University, Busan 621-749, Korea

내분비 장애물질 측정을 위한 붕어 비탈로제닌 정량 분석 ELISA 시스템 개발

이춘일, 김광태, 김안드레, 정규혁[1], 김동규[2], 강신원, 박장수[※]

부산대학교 화학과 신개념 바이오 피지오 센서기술 연구센터, [1]성균관대학교 약학대학, [2]인제대학교 화학과 바이오 헬스 소재 연구센터

요 약

난황단백질인 비탈로제닌을 성숙한 암컷 붕어 혈청으로부터 음이온 교환 크로마토그래피를 이용하여 정제 하였다. 정제한 비탈로제닌을 BALB/c mice를 이용하여 폴리크로날 항체를 생산하였고 이를 protein A column을 사용하여 정제하였다. 또한 이렇게 정제된 폴리크로날 항체를 이용한 붕어 비탈로제닌 측정용 효소면역측정법을 개발하였으며 그 측정 범위는 2~1,000 ng/mL이고 recovery 변동 범위는 88~112%였다. 또한 이 효소면역측정법을 평가하기 위해 성숙한 수컷 붕어를 1,000 ng/L ethinylestradiol (EE_2)에 4주 동안 노출시켜 유도되어지는 비텔로제닌을 측정하였다. 그 결과 성숙한 수컷 붕어의 경우 비탈로제닌이 3주 만에 암컷 붕어의 평균수치만큼 유도됨을 알 수 있었다.

Key words : vitellogenin, ELISA, goldfish, polyclonal antibody

INTRODUCTION

Vitellogenin (VTG), an estrogen induced yolk-precursor phospholipoprotein, is present in the blood of oviparous vertebrates and invertebrates during vitellogenesis. During vitellogenesis, the liver of female oviparous vertebrates and invertebrates is stimulated to produce VTG (Tolar *et al.*, 2001). VTG is synthesized and secreted by the liver in response to circulating estrogens in female oviparous vertebrates and invertebrates. VTG is normally undetectable in the blood of male and immature fish, because the presence of VTG in blood is in a very low concentration (Sole *et al.*, 2001). In other wards, VTG is too low to induce the expression in male and immature.

※ To whom correspondence should be addressed.
Tel: +82-51-510-2294, E-mail: jaspark@pusan.ac.kr

However, male fish also carry the VTG gene and exposure to both natural and synthetic estrogens can trigger its expression (Sumpter and Jobling, 1995). Fish VTG has become a major concern as a useful biomarker for estrogenic compounds in aquatic area (Castillo and Barcelo, 1997; Tyler and Routledge, 1998). Within international bodies such as the Organization Economic Cooperation and Development (OECD) work is ongoing to develop screening and testing programmes for endocrine disrupting effects of new chemicals, and in the focus of this development are the fish test species common species common carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) (Nilsen *et al.*, 2004). Although these fishes can live with polluted environments, fathead minnow, zebrafish and Japanese medaka are all small and low degree in food cycle, not suitable to reflect the food chain accumulation effect. In winter, the carp becomes torpid, retires to the bottom and stops feeding, and Japanese medaka's life cycle does not exceed one year (Gwinn *et al.*, 1992). VTG of hardy goldfish has the advantage to evaluate the estrogenic activity in wild environments in that the fish is omnivorous, feeding on plants, small animals and small crustaceans and other foods, and can reflect the food chain accumulation effect. In addition, it can tolerate many kinds of environments and the life cycle is about 20 years. In winter, it has not a period of dormancy. The fish also has a wide distribution throughout the world. The goldfish is abundant in Korea, Japan, Siberia and China and becomes naturalized in many parts of the eastern U.S.

MATERIALS AND METHODS

1. Fish and vitelloginin induction

Adult female (n = 5) and male (n = 3) goldfish, with an average mass of 200 g, were bought from the local market. To obtain a serum with high vitellogenin concentrations in goldfish, adult female goldfish were anesthetized with a solution of MS22 (Sigma) at a concentration of 0.2 g/L and injected with a intraperitoneal dose of 500 μg/kg EE_2 on 0 and 7day. After 14days exposure they were anesthetized with MS22 (0.2 g/L), blood was collected by cardiac puncture. Male was not treated as a control. To prevent proteolysis, 4 TIU/mL aprotinin were added, then the samples were obtained by centrifugation at 3000 g for 10 min at 4°C. The samples was stored at −20°C.

2. purification and storage of vitelloginin

VTG was purified as previously described (Montorzi *et al.*, 1994). Aliquots of serum were adjusted to contain 35% saturated, followed by centrifugation at 5000 g for 1 min. The supernatant was dialyzed against 25 mM Hepes, 1 mM PMSF, and 1 μM leupeptin, pH 7.5 and 100 μL aliquots were applied to a Mono-Q column, HR 5/5 (Pharmacia), equilibrated with 25 mM Hepes, pH 7.5 at 4°C. The sample was eluted with a linear gradient ranging from 0 to 1 M NaCl in 25 mM Hepes buffer, pH 7.5. 0.5 mL fractions were collected at a flow rate of 2 mL/min, and the elution was monitored at 280 nm.

The purified-VTG was confirmed for purity, molecular mass and character by comparison with plasma proteins using 10% SDS-PAGE gels. The concentration of the purified protein was determined according to the lowry method, using bovine serum albumin as standard protein. The purified-VTG was used as an antigen for anti-VTG antibodies.

3. Immunization and polyclonal antibody production

Female BALB/c mice were immunized with intraperitoneal injections of the purified goldfish VTG. Briefly, 100 μg (500 μg/mL) of VTG was diluted 1 : 1 in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the subsequent injections and administered subcutaneously three times at 3-week intervals. Two weeks after the third injection, the mice received an intraperitoneal injection of 100 μg of VTG and all mice were bled from

the tail 1 week after each booster injection. Blood was centrifuged at 2000 g for 10 min, then stored at −20°C. Antibody was purified with an immobilized protein A column.

4. ELISAs detecting goldfish vitelloginin

NUNC-immuno™ plate were precoated with Goldfish VTG or serum overnight at 4°C with 50 μL (50 μg/mL). Wells containing PBS buffer were used a control. After washing three times with PBST buffer (PBS with 0.05% Tween 20), the wells were blocked for one hour at 37°C with 100 μL of PBSTG (PBST with 1% Gelatin). The wells were washed with PBST buffer, then 100 μL polyclonal antidody was added. After two hours incubation, the plates were washed three times with PBST buffer and 20 μL of a goat anti-mouse IgG conjugated with alkaline phosphotase (1 : 5000). After two hours the plates were washed three times with PBST buffer again and 50 μL phosphatase substrate solutions were added. The enzyme substrate reaction proceeded for about 10 min and stopped by 2.5% ethylene diamine tetraacetic acid (EDTA). Absorbance was read with a ELISA reader at 595 nm.

5. Validation for quantitative gold fish vitelloginin ELISA system

Recovery was assessed by adding purified VTG to control male serum sample at ratio 1 : 9 diluted in PBS 1 : 500, 1 : 1000 and 1 : 2000. The measured and expected values of the samples were compared (Fig. 3).

6. Evaluation of the vitelloginin ELISA system

Adult male (n = 20) and female (n = 5) Goldfish were purchased from a local market and kept in 500 L tanks. The tanks were fitted with recycling filtered and dechlorinated water and kept at a controlled temperature of 15°C. Fish was fed ad libitum with a commercial diet (DIABAQ, DIPROTEG, S.A.). To evaluate the performance of the VTG ELISA system, 20 Goldfish was treated with EE_2 at concentrations of 1000 ng/L and 5 female was served as a positive control. Goldfish was cultured for 4 weeks and every 1 week, the blood was withdrawn from caudal vein of the live fish with a syringe containing 4 TIU/mL aprotinin, transferred to a sterilized tube and centrifuged at 3000 g for 10 min to separate serum, and the serum was stored at −20°C.

RESULT

Goldfish VTG was purified from EE_2 treated female for immunization as the antigen in polyclonal antibody production. The chromatography profile for purification of VTG was shown in Fig. 1. The VTG was specially induced by EE_2 in female but not observed in control male serum. Two bands with molecular weights of 150 Kda and 160 Kda were showed by 10% SDS-PAGE (Fig. 2). The standard curve was established using twelve dilutions of purified VTG. The linear part of the curve corresponded to concentrations ranged from 2 ng/mL to 1000 ng/mL (Fig. 3). The recovery was obtained at 1 : 1000 dilution sample and ranged from 88% to 112% (Fig. 3). The male VTG induced with EE_2 was significantly increased after 7 days exposure, but this increase was

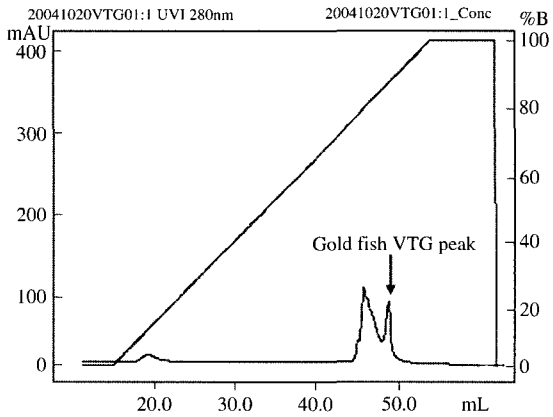

Fig. 1. Purification of goldfish VTG from E_2-treated female fish by anion-exchange chromatography on a MonoQ column. Elution profile of proteins is shown by the solid line, and a gradient of elution buffer (1.0 M NaCl) by the dotted line. Arrow indicates the VTG peak.

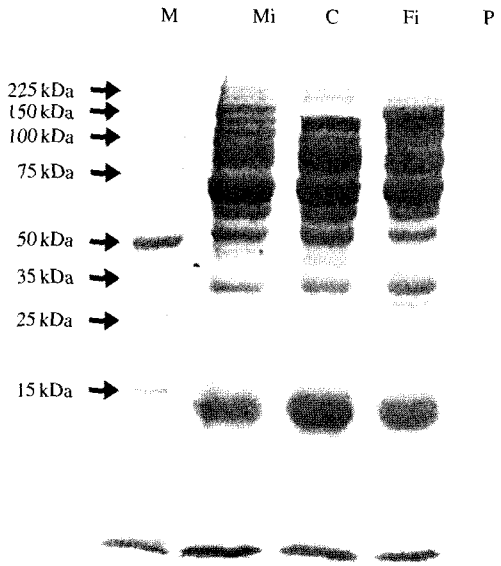

Fig. 2. 10% SDS-PAGE of EE_2 induced male serum (Mi), EE_2 induced female serum (Fi), purified goldfish VTG (P), control male serum (C), and molecular weight standard (M). molecular weight of each band indicated on the left side. The gel was stained with 0.1% coomassie brilliant blue.

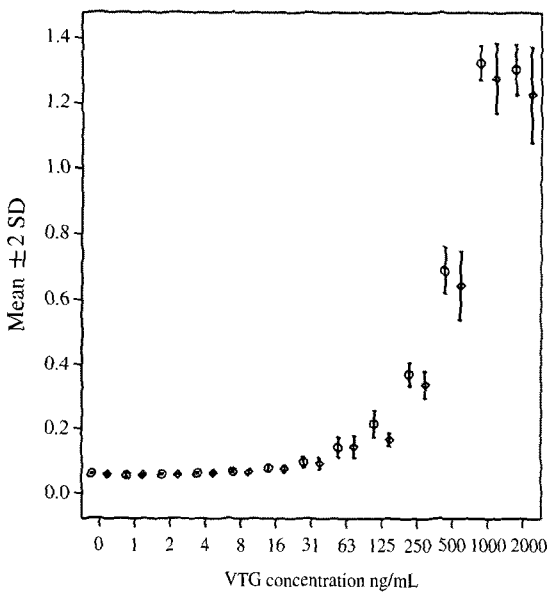

Fig. 3. Comparison of recovery dilution curve of samples (◇) and standard curve (○) by using twelve dilutions of the purified VTG. Each value indicates the means ±2 S.D.

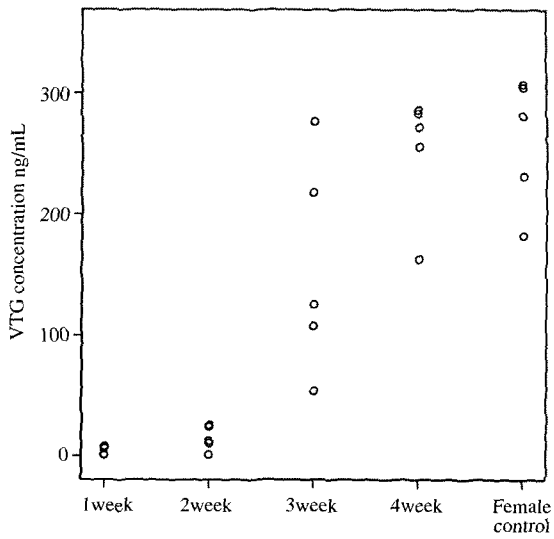

Fig. 4. Change of the VTG levels in male goldfish exposed to different period of EE_2.

lower than the female positive control ($p < 0.05$). After 14 days, the male VTG level with EE_2 treated was significantly lower than control female ($p < 0.05$). After 3 weeks, male VTG level increase up to female levels ($p < 0.05$) and Male goldfish VTG concentration ranged from 163 to 286 μg/mL (Fig. 4).

DISCUSSION

The purification of VTG was performed using ion exchange chromatography and the purified protein was characterized by 10% SDS-PAGE. Two bands with molecular mass of 150 Kda, 160 Kda respectively were observed in female serum specially but not observed in male serum. A previous report showed that carp VTG was unstable and easily degraded (Tyler and sumpter, 1990), thus two similar bands might be a dimmer with two subunits of 150 KDa and 160 KDa.

Contaminated industrial effluents often contain a variety of organic pollutants that can disturb the development of the endocrine system and the organs that respond to endocrine signals in organisms. Environmental monitoring generally requires the analysis of a large number of samples; therefore, there is a

need to search for low-cost, rapid and automated methods. In the recent studies, one of the most developed field testing methods has been enzyme-linked immunosorbent assays (ELISA). This study describes the development and validation of an ELISAs for goldfish vitellogenin, using polyclonal antibody against goldfish VTG. The assay ranged from 2 ng/mL to 1000 ng/mL. The available range was comparable with values reported for VTG ELISA of other fish using polyclonal antibody, including for perch 40～960 ng/mL, for carp 27～1730 ng/mL (Hennies *et al.*, 2003), and 0.2～20 ng/mL (Holbech *et al.*, 2001). VTG has been proposed as an ideal biomarker for screening the estrogenic activities of endocrine disruptors in a wide range of oviparous animals. In our experiment, male goldfish was induced by EE_2 and the levels were up to adult female within 3 weeks. So the goldfish is suitable as a biomarker to evaluate the estrogenic activity in freshwater.

CONCLUSION

The ELISAs developed in this study is suitable to evaluate estrogenic activity freshwater using goldfish VTG and simple, easy and low coast for large scale screening. The assay has been evaluated with EE_2 induced male goldfish and the detection range is wide enough to evaluate goldfish VTG level.

ACKNOWLEDGEMENTS

This research was supported by the grant from the Korea Health 21 R & D project, Ministry of Health & Welfare, Republic of Korea (02-PJ3-PG6-EV05-0001).

REFERENCES

Castillo M and Barcelo D. Analysis of industrial effluents to determine endocrine-disrupting chemicals. Trac-Trend. Anal. Chem. 1997; 16(10): 574-583.

Gwinn RP, Norton PB and Geotz PW. The new encyclopadia britannica. Encyclopadia Britannica, Inc. Chicago. 1992.

Hennies M, Wiesmann M, Allner B and Sauerwein H. Vitellogenin in carp (*Cyprinus carpio*) and perch (*Perca fluviatilis*): purification, characterization and development of an ELISA for the detection of estrogenic effects. Sci. Total. Environ. 2003; 309(1-3): 93-103.

Holbech H, Andersen L, Petersen GI, Korsgaard B, Pedersen KL and Bjerregaard P. Development of an ELISA for vitellogenin in whole body homogenate of zebrafish (*Danio rerio*). Comp Biochem Physiol C Toxicol Pharmacol. 2001; 130(1): 119-131.

Montorzi M, Falchuk KH and Vallee BL. Xenopus-laevis vitellogenin is a zinc protein. Biochem. Bioph. Res. Co. 1994; 200(3): 1407-1413.

Nilsen MV, Berg K, Eidem JK, Kristiansen SI, Brion F, Porcher JM and Goksoyr A. Development of quantitative vitellogenin-ELISA assays for fish test species used in endocrine disruptor screening. Anal. Bioanal. Chem. 2004; 378(3): 621-633.

Sole M, Porte C and Barcelo D. Analysis of the estrogenic activity of sewage treatment works and receiving waters using vitellogenin induction in fish as a biomarker. TracTrend. Anal. Chem. 2001; 20(9): 518-525.

Sumpter JP and Jobling S. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. Environ. Health. Persp. 1995; 103(7): 173-178.

Tolar JF, Mehollin AR, Watson RD and Angus RA. Mosquitofish (Gambusia affinis) vitellogenin : identification, purification, and immunoassay, Comp Biochem Physiol C Toxicol Pharmacol, 2001; 128(2): 237-245.

Tyler CR and Sumpter JP. The purification and partial characterization of carp, (cyprinus-carpio), vitellogenin. Fish. Physiol. Biochem. 1990; 8: 111-120.

Tyler CR and Routledge EJ. Oestrogenic effects in fish in English rivers with evidence of their causation. Pure. Appl. Chem. 1998; 70(9): 1795-1804.