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Development of Quantitative Vitellogenin ELISAs for Bullfrog (*Rana catesbeiana*) used in Endocrine Disruptor Screening

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내분비계 장애물질 검색을 위한 효소면역측정법을 이용한 황소개구리 비텔로제닌 정량법 개발

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요 약

난생 생물의 알 생성유도 단백질인 비텔로제닌(vitellogenin, VTG)을 성숙한 암컷 황소개구리(*Rana catesbeiana*) 혈청으로부터 음이온 교환 크로마토그래피를 이용하여 정제 하였으며 정제한 비텔로제닌을 BALB/c mice에 주사하여 폴리크로날 항체를 생산하였고 이것은 protein A column으로 정제 하였다. 이렇게 정제된 폴리크로날 항체를 이용하여 황소개구리 비탈로제닌 측정용 효소면역측정법을 개발하였으며 그 측정 범위는 12~1,560 ng/mL였다. 또한 이 효소면역측정법을 평가하기 위해 성숙한 수컷 황소개구리를 청정지역과 폐수처리장 하류 하천에서 서식하는 황소개구리 혈액 속의 비텔로제닌을 측정하였다. 그 결과 폐수처리장 하류 하천에 서식하는 수컷 황소개구리 비텔로제닌이 청정지역보다 현저하게 높게 유도됨을 알 수 있었다.

Key words : *Rana catesbeiana*, vitellogenin, ELISA, polyclonal antibody

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INTRODUCTION

Vitellogenin (VTG), an estrogen induced yolk-precursor glycophospholipoprotein, 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 (Sole *et al.*, 2001). VTG is normally undetectable in the blood of male because the presence of VTG in plasma is in a very low concentration. In other words, VTG is too low to detect the expression in male. However, it can be induced by estradiol injection. Exposure of male to estrogenic compounds can be triggers of VTG production (Sole *et al.*, 2001). The presence of VTG in blood of male has been selected as a biomarker to investigate estrogenic activity in amphibians (Mitsui *et al.*, 2003). Bullfrog VTG can be served as a useful biomaker for detecting the level of exposure estrogenic chemicals.

In this study, we isolated bullfrog VTG from the serum of female bullfrog. Bullfrog VTG was purified with Mono-Q column. The purified protein was used as antigen for antibody production and polyclonal antibody was produced against bullfrog VTG. We developed Enzyme linked immunosorbent assay (ELISA) for quantification of bullfrog VTG using this polyclonal antibody. Effluent from Sewage treatment works (STWs) contains estrogenic substances and can induce VTG in fish (Tyler and Routledge, 1998). Using this ELISA, we carried out an assay which has been developed to screen for estrogenic activity of chemicals in STWs.

MATERIALS AND METHODS

1. Frog and vitelloginin induction

Sexually mature female (n=5) and male (n=3) bullfrog, each approximate of 200 g, was purchased from local market. To obtain serum with high level of bullfrog VTG, 17β-estradiol (EE_2) was injected to bullfrog with a intraperitoneal dose of 500 μg/kg every 7 days. After 14 days exposure they were anesthetized with MS222 (0.2 g/L), blood was collected by cardiac puncture. Male (n=2) was not treated as a control. To prevent proteolysis, 4 TIU/mL aprotinin were added, then the samples were obtained by centrifugation at 3,000 × g for 10 min at 4°C. The sample was stored at −20°C.

2. Purification and storage of vitelloginin

1 mL of female bullfrog serum was adjusted to be saturated 35% $(NH_4)_2SO_4$ solution maintained at 0°C and followed by centrifugation at 5,000 × g. The supernatant was dialyzed against 25 mM HEPES, 1 mM PMSF, and 1 μM leupeptin, pH 7.5 for 12 h. After dialysis, the supernatant was applied to Mono-Q column HR 5/5, 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.

VTG purity was confirmed by electrophoresis in denaturing condition. 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 antibody production.

3. Immunization and polyclonal antibody production

Polyclonal antibody was raised in female BALB/C mice (5～6 weeks old) against purified VTG from bullfrog, using standard immunological methods. To start immunization against bullfrog VTG, 100 μg of VTG was diluted 1 : 1 in complete Freund's adjuvant for the first injection. This mixture was injected subcutaneously into three mice. Three additional booster injections followed at 4-week intervals using Freund's incomplete adjuvant. Two weeks after the third injection, the mice received an intraperitoneal injection of 100 μg of VTG and 3 days later they were

sacrificed Blood was centrifuged at 2,000 × g for 10 min and stored at −20°C. Antibody was purified with an immobilized protein A column. After the immunization, the polyclonal antibody was characterized by ELISA.

4. ELISAs detecting bullfrog vitelloginin

An Enzyme-Linked Immunosorbent Assay System was developed to quantitatively detection of bullfrog VTG as the methods described by Li *et al.* (2004). The purified VTG was added to control male serum diluted in PBS 1 : 1,000 and the standard curve was generated with 12 dilutions of VTG.

5. Evaluation of the vitellogenin ELISA system

To investigate correlation of VTG levels and estrogenic compound in aquatic environment, 50 bullfrog male were randomly sampled respectively from both a pristine area and a river next to STW (river STW) over the reproductive period from May to July. Immediately after collection, the serum was collected as described above. The gonads were removed by dissection and weighed. GSI was calculated as follows: GSI=(total gonad weight/total body weight) × 100%.

RESULTS

Bullfrog VTG was purified with Mono-Q column, and VTG was eluted with a linear gradient ranging from 0 to 1 M NaCl. At 0.63 M NaCl, VTG was eluted (Fig. 1). Serum from untreated male, EE_2 treated female, EE_2 treated male and purified VTG were analyzed by denaturing electrophoresis and stained using coommassie brilliant blue. Two bands, 230 kDa and 235 kDa, were shown in EE_2 treated female serum, EE_2 treated male serum and purified VTG, and was identified as bullfrog VTG (Fig. 2). The

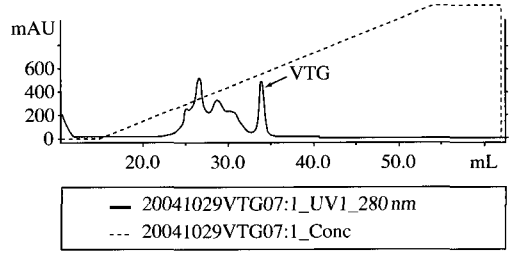

Fig. 1. Purification of bullfrog VTG from EE_2-treated female bullfrog by anion-exchange chromatography on a Mono-Q column. Arrow indicates the VTG peak.

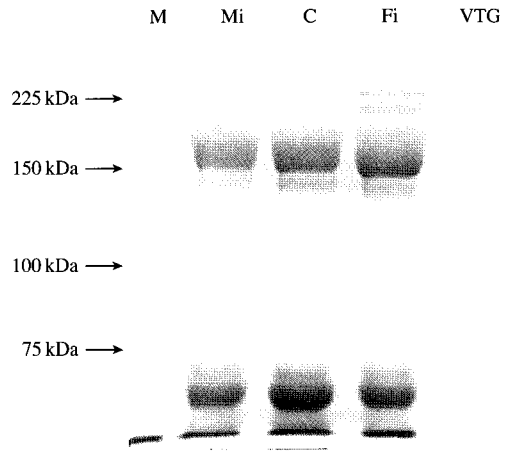

Fig. 2. 6% SDS-PAGE of EE_2 induced male serum (Mi), EE_2 induced female serum (Fi), purified bullfrog VTG (VTG), control male serum (C), and molecular weight standard (M). Molecular weight of each band indicated on the left side. The gel was stained with 0.1% coomassie brilliant blue.

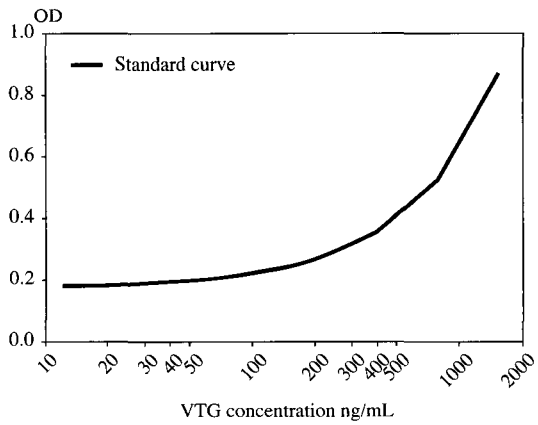

Fig. 3. Standard curve by using eight dilutions of the purified VTG.

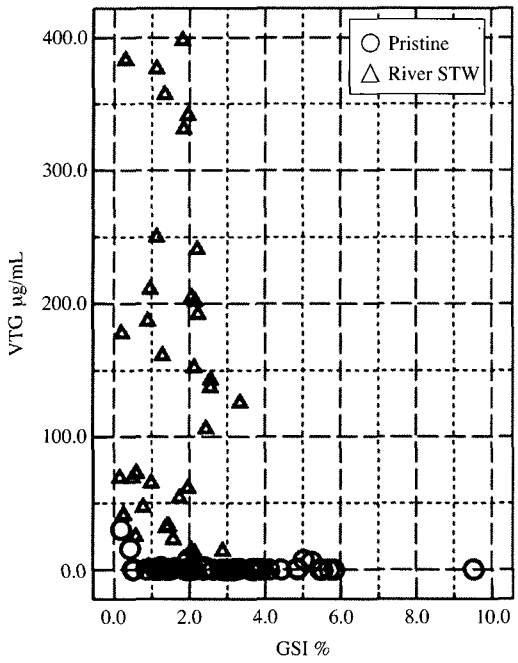

Fig. 4. The correlation between VTG and GSI were shown in male bullfrog from the pristine areas and the river STW.

standard curve was established using 12 dilutions of purified VTG. The linear part of the curve corresponded to concentrations ranged from 12 ng/mL to 1,560 ng/mL (Fig. 3).

The VTG levels of male were more persuasive than female for investigation of estrogenic activity. In contrast, VTG levels from river STW were showed higher levels of VTG almost over all samples. VTG levels from the pristine ranged from 0 μg/mL ~ 30.1 μg/mL and from river STW ranged 0 ~ 398 μg/mL. In the samples of VTG levels > 325 μg/mL from river STW, the GSI was less than 2.0% and in the samples of VTG levels > 100 μg/mL, the GSI was less than 4.0% (Fig. 4).

DISCUSSION

Two forms of VTG were purified from xenopus laevis with Mono-Q column, each having a molecular weights of 220 kDa (Montorzi *et al.*, 1994) and Bullfrog-VTG also showed similar molecular weights. Two different bands, 230 kDa and 235 kDa, were observed in EE_2-treated female serum, EE_2-treated male serum and purified VTG but not observed in control EE_2-untreated male serum.

Concern about the health of aquatic fauna living in waters containing biologically active levels of estrogenic compounds is particularly focused on the effects on their reproductive success (Sole *et al.*, 2002). VTG have been selected as an ideal biomarker to evaluate estrogenic activity in aquatic environments. VTG of various fishes have been used to assess estrogenic endocrine disrupting effects of new chemicals, such as carp (*Cyprinus carpio*), fathead minnow (*Pimephales promelas*), zebrafish (*Danio rerio*) and japanese medaka (*Oryzias latipes*) (Nilsen *et al.*, 2004). Although sandwich ELISAs for quantification of xenopus laevis VTG has been developed (Mitsui *et al.*, 2003) xenopus laevis VTG is not suitable to monitor Korean water environment. Xenopus laevis is from africa and isn't distributed in Korea. bullfrog can live in high polluted environments as amphibians and widely distributed throughout Korea. Hence bullfrog VTG was selected to evaluate aquatic environments. In this study, the ELISAs detection range is from 12 ng/mL to 1,560 ng/mL and is sufficient to monitor aquatic environments. The estrogenicity of the rivers was more evident at the location downstream from STW by elevated levels of VTG in male carp (Sole *et al.*, 2002). An inverse correlation was observed between plasma VTG and GSI in adult male fish roach (*Rutilus rutllus*) (Jobling *et al.*, 1998). Present study showed a trend the lower GSI was corresponding to the higher VTG level and VTG levels of male bullfrog captured from river STW were also significantly higher than from pristine areas as expected. The bullfrog VTG is suitable as a biomarker to evaluate the estrogenic activity in Korea freshwater.

CONCLUSION

The ELISAs for quantification of bullfrog VTG is

suitable to evaluate estrogenic activity in freshwater and simple, easy and low coast for large scale screening. The assay has been evaluated with water of river STW inducing male bullfrog VTG and the detection range is wide enough to evaluate bullfrog VTG level.

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