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Title	Molecular Biological and Immunobiochemical Studies of Multiple Vitellogenins in Dojo Loach (<i>Misgurnus anguillicaudatus</i>) : Integration of Basic Information to be Practical Biomarkers for Evaluating Aquatic Estrogenic Activities [an abstract of dissertation and a summary of dissertation review]
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学 位 論 文 題 目

Molecular Biological and Immunobiochemical Studies of Multiple Vitellogenins in Dojo Loach (*Misgurnus anguillicaudatus*): Integration of Basic Information to be Practical Biomarkers for Evaluating Aquatic Estrogenic Activities

(ドジョウの多型ビテロジェニンに関する分子生物学的及び免疫生化学的研究:
水圏エストロゲン活性評価の実用的バイオマーカーとしての基礎的情報の集積)

In the past decades, increasing attention has been given to evaluate adverse effects of endocrine disrupting chemicals (EDCs) in aquatic environments. Among EDCs, xenoestrogens received major attention owing to their capability to mimic natural estrogens (estrogen mimics). Abundant evidence from field and laboratory studies revealed that exposure to these chemicals has lead the abnormal modulation or disruption of physical development, reproduction and sexual behavior in aquatic wildlife. It is necessary especially for field surveys to predict total endocrine disrupting potency of EEDCs because many chemicals are present as complex mixtures with other compounds; with this regard, measurements of estrogen-sensitive gene transcripts and/or proteins in bio-indicator species (e.g., fishes) have been often used for evaluating the total estrogenic activity contaminated in the aquatic environments. Among such biomarkers, vitellogenin (Vtg), which is a major estrogen-sensitive, female-specific, liver-derived yolk protein precursor, has been utilized most frequently; the abnormal presence of this female-specific marker in male and/or juvenile fishes indicate an estrogenic potency in the aquatic environment where they are inhabiting or exposed.

The dojo loach (*Misgurnus anguillicaudatus*), a demersal freshwater fish, widely distributes in East Asia. Owing to the moderate size, long lifetime and sensitivity to aquatic pollutants, this species is considered to be an appropriate model animal for the aquatic

toxicology studies. However, characterization of *vtg* gene transcripts and Vtg proteins has been incompleting in the dojo loach; based on recent advances on the knowledge of fish *vtg*/Vtg, such characterization has to be performed by considering not single, but multiple *vtg*/Vtg subtypes in the targeted species prior to their use in EEDC surveys.

The primary goal of the present study is to survey the impact of EEDCs contamination in freshwater environments in East Asia by establishing a research model that utilizes Vtg gene transcript (*vtg*) and the corresponding protein product of the dojo loach as an estrogen-sensitive biomarker. To achieve this primary objective, the present study specifically focused on molecular biological and biochemical characterization of loach *vtg*/Vtg, as well as on characterization of their response to various estrogenic stimulants (e.g., model estrogenic chemicals and river waters with potential estrogenicities). A preliminary, but practical, field survey was also conducted to evaluate the current estrogenic potency of two fresh-water environments in China.

Firstly, seven full-length cDNAs (*vtg1*~*7*) encoding two distinct types of Vtg were cloned and characterized in the dojo loach. Type I (Vtg1~6) contained two major portions (lipovitellin heavy chain and phosvitin) but largely lacked the remaining C-terminal portions; type II (Vtg7) lacked both phosvitin and such C-terminal portions. Phylogenetic analysis revealed that six type-I Vtgs (Vtg1~6) were structurally classified as VtgAo1 paralogs, while the type-II Vtg (Vtg7) was classified as VtgC, respectively.

To develop and utilize immunoassays of Vtg proteins, characterization and identification on the subtypes of Vtg proteins were performed. Recombinant loach VtgAo1 and VtgC were induced by a bacterial expression system, purified, and separately injected into two rabbits to obtain two distinct antisera against loach VtgAo1 (a-VtgAo1) and VtgC (a-VtgC), respectively. Western blot analysis indicated that VtgAo1 is the dominant Vtg subtype in the dojo loach, representing its potential to be a suitable estrogen-sensitive marker; meanwhile VtgAo2 and VtgC were considered to be the minor ones or not present in this species.

The quantitative real time reverse-transcription polymerase chain reaction (qRT-PCR) for the quantification of dojo loach total *vtgAo1* transcripts was developed using the universal primer set for *vtgAo1* subtype. Estrogen-inducibility of *vtgAo1*/VtgAo1 with natural estrogen (17 β -estradiol: E2) and synthetic xenoestrogen (17 α -ethynylestradiol: EE2) in the male

dojo loach was investigated at different doses and time intervals. Both *vtgAo1* mRNA and VtgAo1 protein were found to be inducible within a week in male loach following aqueous exposure to EE2 and E2. Inductions of *vtgAo1* gene transcript were found to be about x10 sensitive by comparison with lowest-observed-effect concentration (LOECs) of estrogenic activities than VtgAo1 protein. The expression profiles of *vtgAo1* mRNA in the male loach exhibited a dose-dependent and time-dependent increase following exposure to both estrogens. These findings suggest that the dojo loach is a promising practical model organism to evaluate estrogenic activities in aquatic environment by using *vtgAo1*/VtgAo1 biomarkers.

A preliminary immersion test using hepatic *vtg* expressions in male loach was performed for detecting estrogenic activities of two natural river (the Odajima River and the Tokiwa River) waters in Hakodate, Japan. The result indicated a potential, albeit weak, estrogenic activity in these rivers. No significant difference was found between the mean levels of both river groups. A preliminary, but practical field survey was also conducted to evaluate the current estrogenic potency of two fresh-water environments (Nicheng and Rudong) in China by evaluating the *vtg* expression, as well as histological observations of the gonadal morphology in wild loach. All male fish appeared to be positive for *vtgAo1* synthesis, indicating a potential estrogenic activity in the survey areas. The mean of *vtgAo1* expressions in female fish caught from Rudong was significantly higher than that of Nicheng group perhaps due to their difference in ovarian development. No abnormal gonad, such as testis-ova, was found in any male fishes.

In conclusion, multiplicity of *vtg*/Vtg (i.e., *vtgAo1*/VtgAo1 and *vtgC*/VtgC) appeared to be evident in dojo loach. The major *vtg*/Vtg type was found to be *vtgAo1*/VtgAo1. Exposure tests with estrogens and river waters, in conjunction with preliminary field surveys, confirmed that the dojo loach has the potential to be a test model for monitoring the estrogenic activities in the aquatic fresh water environments across East Asian countries by utilizing *vtgAo1*/VtgAo1 of this species as the marker of estrogenic exposure.