Korean J. Limnol. 41 (4): 541~546 (2008)

EROD and TOSC Assays Using Sentinel Fish Species as Tools for Assessing Physiological Level of Aquatic Ecosystem Health: Case Study

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The purpose of this study was to evaluate ecosystem health effect in the physiological levels, based on ethoxyresorufin-*O*-deethylase (EROD) and total oxyradical scavenging capacity (TOSC) assays using sentinel fish species. We collected fish samples of *Zacco platypus* in May 2008 from 3 sampling sites including upstream, midstream, and downstream of the Gap Stream. EROD activity was averaged 4.54 in the downstream, 2.7 fold higher than upstream and indicated that stream condition was degraded along with longitudinal gradient from up to downstream. Downstream, especially was significantly increased ($p<0.01$) so that indicated various pollutants including nutrient enrichment and toxicant exposure from the point sources, wastewater treatment plant and industrial complex may impact to the stream condition. In the mean time, TOSC assays showed higher in the midstream than other sites, but the values were not significant, compared to the previous report on oxidative stress. Overall results indicated that our approaches applying two biomarkers can be effectively used for diagnosis of the physiological levels in an integrative stream health assessments and can be applied as useful pre-warning techniques as a biochemical alarm system of organic pollutions.

Key words : EROD assay, TOSC assay, freshwater fish, water pollution

INTRODUCTION

Since 1970s, stream ecosystem in Korea have been primarily influenced by rapid urbanization, mainly in associated with high human activities by dense populations (Lee and An, 2007). Especially, enormous chemical compounds to make human more comfortable to live usually inflow to the stream ecosystem and have been caused many side effects instead of their original purpose. These chemicals have been affected to not only pollute the ecosystem but furthermore, induced endocrinical disorder to cause reproductive failures of human being and wildlifes (Colborn *et al.*, 1993; Jeon *et al.*, 2004). To assess these effects to the stream ecosystem, chemical water quality criteria developed through laboratory toxicity tests on standard test organisms have been traditionally used as surrogates for determining attainment of the biologically based goal of water quality (Adams, 2002).

Recently, health assessments of stream ecosystems have been conducted in various levels including community, population, and individual levels (Adams, 2002). Community level-assessments is

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popular in the health assessments since Index of Biological Integrity (IBI) using fish assemblage concepts was introduced by Karr (1981), and many countries including Korea applied the modified model to assess ecological health of their regional stream ecosystems. This concepts is the integrative ecosystem health evaluation techniques to assess the living biota such as fish in the freshwater ecosystem out of physiochemical assessments, traditional evaluation technique for freshwater quality (An *et al.*, 2006).

Adams (2002), however, pointed out that community-level evaluation technique shows slow response against water pollution and can detect the condition which pollution proceed over some severe levels as ecological significance. For this reason, biomarkers based on mechanistic basis, have developed and proved to be a useful tool in detecting early biological changes caused by environmental pollution. These biomarkers are defined as sensitive, measurable, xenobiotically induced changes in biochemical processes (e.g. enzyme activity) (Peakall, 1992). In particular, ethoxyresorufin-*O*-deethylase (EROD) and total oxidant scavenging capacity (TOSC) assay were usually applied to evaluate physiological effects in stream ecosystem. For the assay, the sentinel fish species, *Zacco platypus* was chosen because it is the most abundant and widely distributed species in Korea and also one of the most dominant species in Korean streams. The presence of hepatic EROD enzyme induction in fish is an indication of the exposure of the organism to potentially toxic compounds, such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Burgeot *et al.*, 1996). Providing evidence of receptor-mediated induction of cytochrome P450-dependent monooxygenases (the CYP1A subfamily specifically) by xenobiotic chemicals, EROD is a highly sensitive indicator of contaminant exposure in fish (Whyte *et al.*, 2000). TOSC assay is to measure and quantify the capacity of animals to neutralize reactive oxygen species (ROS-superoxide anion, hydrogen peroxide, hydroxyl radicals, peroxyl radicals), thus providing an index of biological resistance to ROS (Winston *et al.*, 1998). This assay has been used to evaluate the ROS scavenging capacity of tissues from a number of adult marine invertebrate species (Regoli and Winston, 1998; Regoli *et al.*, 2000) and applied as good biomarker of the oxidative stress, measuring antioxidative reaction in the field of ecotoxicology (Regoli *et al.*, 2002b; Aruoma, 2003; Kim *et al.*, 2005; Bocchetti and Regoli, 2006). Approach of TOSC assay has a great predictive value on the health condition of the aquatic organisms and allows to discern the various roles of specific ROS in oxidative stress syndrome (Regoli *et al.*, 2002b). In this study, we applied two biomarkers as EROD and TOSC assay to assess the physiological stress effects in the freshwater ecosystem using by fish and to evaluate the function as pre-warning system.

MATERIALS AND METHODS

1. Descriptions of field sampling sites

This study was proceeded in the Gap Stream, which is one of main tributaries of the Geum River, especially running through the Daejeon city nearby the downstream reaches so that could be represent as typical urban stream in the downstream region. Sampling sites were selected in 3 site where could be represented as up, mid, and downstream in May 2008, the season with stablized waterbody before the monsoon in summer. All sites were considered by point-sources existence near the location and also analyzed the land use patterns. Upstream site (S1, S=site) was located in outside of urban area, mainly surrounded by over 70% of pristine forest and some paddies and ordinary fields beside the stream. Midstream site (S2) could regard as artificially effected site nearby general human activities occurred by such as hospital, bridges and inflow urban sewages. In addition, artificial stream bank was constructed along with the stream but it was well vegetated, mostly covered by lawn grass. Downstream site (S3) had been moderately impacted severely by organic and inorganic contaminations caused by sewages input from Daejeon industrial complex and wastewater treatment plants, located near the site. All sampling sites are located in Daejeon city and detail sites with the stream order (Strahler, 1957) are as follows:

S1 (upstream): E36° 15′09″, N127° 19′20″, Bongoek 2nd bridge, Bongoek-dong, Seo-gu (3rd order)

S2 (midstream): E36° 20′28″, N127° 21′10″, Manyeon bridge, Wolpyeong-dong, Seo-gu (4th order)

S3 (downstream): E36° 24′17″, N127° 24′47″,

Gapcheon bridge, Jeonmin-dong, Daedeok-gu (5th order)

2. Fish collection and assay preparation

For the fish capture, we used a cast net (mesh 5×5 mm) and kick net (4×4 mm), the most common sampling gears of wadable stream for the fish study in Korea. We choose the sentinel species, *Zacco platypus* as perviously described. In each site, 3 female individuals of sentinel species with a similar size around 11 cm were selected. It was transported to the laboratory being alive with oxygenated water by air supply and dissected living species immediately to obtain 2 samples from fresh liver tissue in each individual for EROD assay and 1 sample for TOSC assay.

3. EROD assay

EROD assay was performed according to the method of Hanioka *et al.* (2000) with some modification on the basis of procedures by Burke and Mayer (1974) and measured fluorometrically in the liver tissue. EROD activities were determined by quantification of the resorufin production from dealkylation of ethoxyresorufin by liver microsomes. The standard incubation mixture contained ethoxyresorufin (200 μM), spiked separately as substrate (dissolved in methanol), liver microsomal proteins from fish (average 10 mg) in a final volume of 400 μL of 50 mM potassium phosphate buffer (pH 7.4). After preincubation at 37°C for 3 minutes, the reaction was started by addition of NADPH (40 mM). The mixture was remained 6 minutes for 37°C for the incubation and was terminated with ice-cold methanol 800 μL with vortexing. After cooling in ice for 15 minutes, the samples were centrifuged at 6,000 g for 20 minutes. The supernatant was filtered and diluted with water by 1 : 2 ratio. Using 50 μL of diluted samples, it was analyzed by HPLC immediately. HPLC analysis was conducted using Shimadzu SCL-10A system controller (Kyoto, Japan).

The column was used by Phenomenex Luna C18 (2) 100 A and temperature was kept at 40°C. The product was eluted isocratically with 20 mM phosphate buffer (pH 6.8)-methanol-acetonitrile (52 : 45 : 3, v/v) at 0.8 mL min^{-1}, flow rate. The excitation and emission wavelength in fluorescence detector were fixed at 560 and 585 nm, respectively. The formation rate of the product from ethoxyresorufin was calculated from the peak area of different concentrations of resorufin.

4. TOSC asssay

TOSC assay was conducted by the methods of Kim *et al.* (2005), on the basis of the approaches by Winston *et al.* (1998), Regoli and Winston (1999), and Regoli *et al.* (2000) and used to evaluate antioxidant behavior of the medium. Basically, TOSC assay is based on the ethylene-yielding reaction of alpha-keto-gamma methiolbutyric acid (KMBA) with peroxyl radicals and peroxynitrite. Peroxyl radicals were produced upon the thermal homolysis of 2,2′- azobis-amidinopropane (ABAP) at 35°C. Peroxynitrite was generated from the decomposition of SIN-1 which oxydized KMBA to ethylene (Kim *et al.*, 2005). The assay conditions used in this experiment were 0.3 mM KMBA and 60 mM, ABAP and 210 μM SIN-1 (3-morpholinosyndnomine) in 100 mM potassium phosphate buffer (pH 7.4). To examine TOSC for fresh medium and medium conditioned by 1 day of culture with hepatocytes from fish liver, both media were diluted 5-fold in distilled water and 0.1 mL of the diluent was added into each vial. The control reaction consisted of 0.1 mL of distilled water instead of diluted medium. Reactions were carried out in 12-mL rubber septum-sealed vials in a final reaction volume of 1 mL. Ethylene production was measured by GC analysis of 0.2-mL aliquots taken directly from the headspace of the reaction vials. Samples were monitored in sequence at 12-min intervals. Analyses were performed with a Hewlett-Packard (Series II 5890) gas chromatograph equipped with a 30 m capillary SPB-1 column (Supelco) and a flame ionization detector. The oven, injection, and detector temperatures were respectively, 60, 180, and 180°C. Nitrogen was used as the carrier gas at a flow rate of 30 mL min^{-1} with 134 k pa^{-1}, injector pressure. TOSC values were quantified from the equation TOSC $=100-(SA/CA\times 100)$, where SA and CA were the integrated areas from the curve that best defined the experimental points during the reaction time course for sample and control reactions, respectively. A sample with no oxy-radical scavenging capacity receives a TOSC value of 0%, because it has the same area under the curve as the control reaction (SA/CA=1). A sample that suppressed the ethylene formation entirely possesses an area under the curve of 0 (SA=0), and thereby, a TOSC

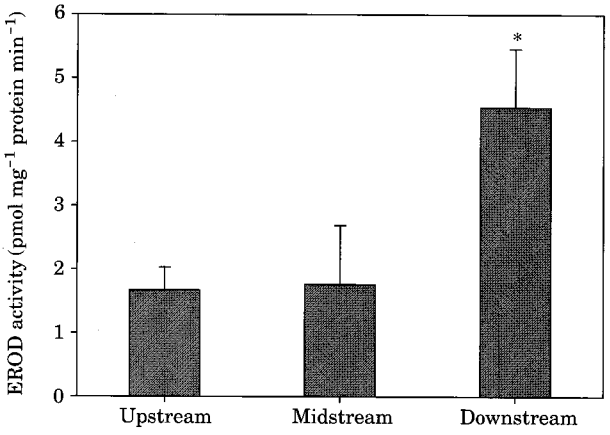

Fig. 1. 7-ethoxyresorufin-*O*-deethylase dealkylation (EROD) activity in *Zacco platypus* from upstream to downstream in the Gap Stream. The asterisk (*) indicates statistically significant differences according to NK-test after ANOVA ($P < 0.05$, n=6).

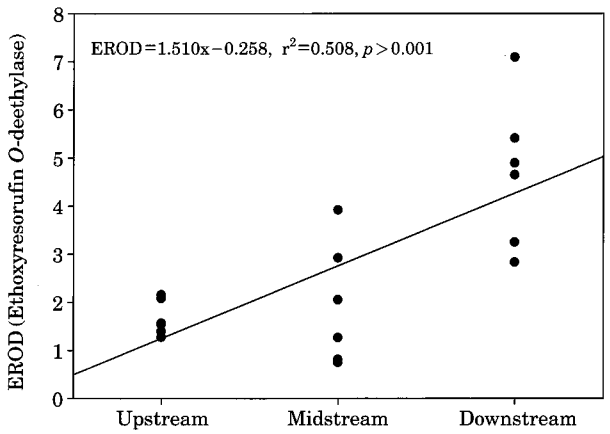

Fig. 2. Regression analysis along the gradient of up to downstream based on EROD activity (n=6).

value of 100%. The specific TOSC was calculated by dividing the experimental TOSC by the volume of medium.

5. Statistical analysis

Significant differences between groups were determined by analysis of variance (ANOVA) test, following by Newman-Keuls comparison tests ($p < 0.05$). All statistical tests were performed under SPSS/Window version 12.0.

RESULTS AND DISCUSSION

According to enzyme activities analysis derived from EROD assay, the magnitude of the response was 2.7 fold higher in the downstream than upstream site of the Gap Stream, and the difference was statistically significant ($p < 0.05$). Also, this response was higher ($p < 0.05$) in the downstream than the midstream area and this was shown well in the ANOVA test (Fig. 1). It also may tend to increase along with the main axis from the upstream to downstream for EROD activity (Fig. 2). EROD assay has been established as a useful biomarker in a number of field investigations of industrial effluents, contaminated sediments and chemical spills (Fouchecart *et al.*, 1999; Whyte *et al.*, 2000). In this study, downstream area was mainly influenced by the water pollution by the inflow from several point sources such as industrial complex and wastewater treatment plants located nearby the stream. Especially, effluents with chemical compounds from those point sources may impact to the downstream ecosystem so that increased EROD activity in downstream indicated that it was exposed to elevate levels of inducers for living biota in downstream area.

Table 1. Total oxyradical scavenging capacity (expressed in TOSC unit mg^{-1} protein mL^{-1}) towards peroxyl radicals of *Zacco platypus* in the Gap Stream (n=3).

Stream sites	Mean	Min.	Max.
S1 (Upstream)	4.8±5.3	1.04	10.83
S2 (Midstream)	16.6±9.9	9.8	28
S3 (Downstream)	6.9±2.3	5.76	9.62

From the analysis of TOSC assay, it was hardly showed some patterns between upstream and downstream as different as EROD assay (Table 1). Especially, it was the highest mean value in midstream instead of downstream, where have point sources nearby the stream. As Regoli *et al.* (2002b) reported that TOSC was less sensitive response but provided more holistic picture of susceptability to the oxidative stress, so midstream, especially could be affected and exposed the oxidative stress because it was located in the place just started effects from human activities in the relation with urbanization. However, values of TOSC in this study was significantly lower than other studies previously reported (Regoli *et al.*, 2002a; Regoli *et al.*, 2005) so that we may regards that the effect of oxidative stress in this study

were less impacted.

Previous researches about the sites in this study reported that nutrients (TP, TN) was significantly high in downstream site with almost 5 fold values compared to upstream area (Lee and An, 2007) in chemical level and also pointed out habitat disturbance was severely occurred by assessment of QHEI (Qualitative Habitat Evaluation Index) in physical level approaches (Bae and An, 2006). One of the most useful tools to evaluate the stream ecosystem health, IBI (Index of Biological Integrity) indicated the degradation of stream health along the gradient of up to downstream reaches was obvious and especially, downstream was severely impacted and scored very poor and poor condition, respectively (An *et al.*, 2001; Bae an An, 2006). In the mean time, EROD and TOSC assay were based on the reactive analysis of liver in fish. Especially, liver have the most important detoxification function in all vertebrates so that it could be key organ to diagnose toxicants effects derived from chemical compounds to the living biota. Especially, liver in fish have the most abundant lipids so that could be the most accumulative organ by organic pollutants. Thus, it was more easy to inhibit the activation of detoxification enzyme for the liver damages from chemical pollutants than other organs (Kime, 1998) so that EROD activity could show the significant chemical influences in downstream.

Followed by results from two biomarker assays, EROD assay could detect the influence at the dowstream area from point sources nearby the stream and had a tendency of degradation along the gradient of up to downstream. downstream area in this study heavily impacted by enriched nutrients from effluents of point sources. However, TOSC assay, the one of the newest approaches to provide a parameter for oxidative stressor (KFDA, 2003), indicated that oxidative stress in the Gap Stream was less influenced. Overall results indicated that our approaches applying two biomarkers can be effectively used for diagnosis of the physiological levels in an integrative stream health assessments and can be applied as useful pre-warning techniques as a biochemical alarm system of organic pollutions.

ACKNOWLEDGEMENTS

This research is supported by the grant "Assessment model developments of biological, chemical, and physical habitat in the level of fish individual and community in aquatic ecosystems and their optimum stressor analysis", the Ministry of Environment, Korea in 2008.

LITERATURE CITED

Adams, S.M. 2002. Biological indicators of aquatic ecosystem stress. Bathesda, American fishery society, 644pp

An, K-G., D.H. Yeom and S.K. Lee. 2001. Rapid bioassessments of Kap stream using the Index of Biological Integrity. *Korean Journal of Environmental Biology* **19**: 261-269.

An, K-G., J.Y. Lee, D.Y. Bae, J.H. Kim, S.J. Hwang, D.H. Won, J.K. Lee and C.S. Kim. 2006. Ecological assessments of aquatic environment using multi-metric model in major nationwide stream watersheds. *Journal of Korean Water Quality* **22**(5): 796-804.

Aruoma, O.I. 2003. Methodological considerations for characterizing potential antioxidant actions of bioactive components in plant foods. *Mutation Research* **9**: 523-524.

Bae, D.Y. and K-G. An. 2006. Stream ecosystem assessments, based on a biological multimetric parameter model and water chemistry analysis. *Korean Journal of Limnology* **39**: 198-208.

Bocchetti, R. and F. Regoli. 2006. Seasonal variability of oxidative biomarkers, lysosomal parameters, metallothioneins and peroxisomal enzymes in the Mediterranean mussel *Mytilus galloprovincialis* from Adriatic Sea. *Chemosphere* **65**: 913-921.

Burgeot, T., G. Bocquéné, C. Porte, J. Dimeet, R.M. Santella, L.M. Garcia de la Parra, A. Pfhol-Leszkowicz, C. Raoux and F. Galgani. 1996. Bioindicators of pollutant exposure in the northwestern Mediterranean Sea. *Marine Ecology Progress Series* **131**: 125-141.

Burke, M.D. and R.T. Mayer. 1974. Ethoxyresorufin: direct fluorimetric assay of a microsomal *O*-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabolism and Disposition* **2**: 583-588.

Colborn, T., F.S. vom Saal and A.M. Sato. 1993. Developmental effects of endorine disrupting chemicals in wildlife and humans. *Environmental Health Perspectives* **101**: 378-384.

Fouchecart, M.O., M. Arnold, P. Berny, B. Sideman, B. Rather and J.L. Riviera. 1999. Assessment of the bioavailability of PAHs in rats exposed to a polluted soil by natural routes: Induction of EROD activity and DNA adducts and PAH burden in both liver and lung. *Environmental Research* **80**: 330-339.

Hanioka, N., N. Tatarazako, H. Jinno, K. Arizono, and

M. Ando. 2000. Determinatiion of cytochrome P450 1A activities in mammalian liver microsomes by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography B* **744**: 399-406.

Jeon, J.K., J.S. Lee, Y.C. Sohn, W.J. Shim, J.H. Jeung, G.P. Hong, P.K. Kim and C.H. Han. 2004. Responses of cytochrome p450 and EROD activity in rockfish (*Sebastes schlegeli*) administered intraperitoneal injection of 4-nonylphenol. *Korean Journal of Environmental Biology* **22**: 171-176.

Karr, J.R. 1981. Assessment of biotic integrity using fish communities. *Fisheries* **6**: 21-27.

Kim, S.K., K.J. Woodcroft, S.J. Oh, M.A. Abdelmegeed and R.F. Novak. 2005. Role of mechanical and redox stress in activation of mitogen-activated protein kinases in primary cultured rat hepatocytes. *Biochemical Pharmacology* **70**: 1785-1795.

Kime, D.E. 1998. Endocrine disruption in fish. Kluwer Academic Publishers, Boston.

Korea Food and Drug Administration (KFDA). 2003. Establishment functionality evaluation system for antioxidant of health functional food. KFDA. Seoul.

Lee, J.H. and K-G. An. 2007b. Seasonal dynamics of fish fauna and compositions in the Gap stream along with conventional water quality. *Korean Journal of Limnology* **40**: 503-510.

Peakall, D. 1992. Animal Biomarkers as Pollution Indicators. Chapman and Hall, London, p. 368.

Regoli, F. and G.W. Winston. 1999. Quantification of total oxidant scavenging capacity (TOSC) of antioxidants for peroxynitrite, peroxyl radicals and hydroxyl radicals. *Toxicology and Applied Pharmacology* **156**: 96-105.

Regoli, F., M. Nigro, S. Bompadre and G.W. Winston. 2000. Total oxydant scavenging capacity (TOSC) of microsomal and cytosolic fractions from Antarctic, Arctic and Mediterrranean scallops: differentiation between three potent oxidants. *Aquatic Toxicology* **49**: 13-25.

Regoli, F. and G.W. Winston. 1998. Application of a new method for measuring the total oxyradical scavenging capacity in marine invertebrates. *Marine Environment Research* **46**: 439-442.

Regoli, F., M. Nigro, M. Benedetti, S. Gorbi, C. Pretti, P.G. Gervasi and D. Fattorini. 2005. Interactions between metabolism of trace metals and xenobiotic agonists of the aryl hydrocarbon receptor in the antarctic fish *trematomus bernacchii*: Environmental perspectives. *Environmental Toxicology and Chemistry* **24**: 1475-1482.

Regoli, F., M. Nigro, M. Chiantore and G.W. Winston. 2002a. Seasonal variations of susceptibility to oxidative stress in Adamussium colbecki, a key bioindicator species for the Antarctic marine environment. *The Science of the Total Environment* **286**: 205-211.

Regoli, F., S. Gorbi, G. Frenzilli, M. Nigro, I. Corsi, S. Focardi and G.W. Winston. 2002b. Oxidative stress in ecotoxicology: from the analysis of individual antioxidants to a more integrated approach. *Marine Environment Research* **54**: 419-423.

Strahler, A.N. 1957. Quantitative analysis of watershed geomorphology. *American Geophysical Union Transactions* **38**: 913-920.

Whyte, J.J., R.E. Jung, C.J. Schmitt and D.E. Tillitt. 2000. Ethoxyresorufin-*O*-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology* **30**: 347-570.

Winston, G.W., F. Regoli, A.J. Dugas Jr., J.H. Fong and K.A. Blanchard. 1998. A rapid gas chromatographic assay for determining oxyradical scavenging capacity of antioxidants and biological fluids. *Free Radical Biology and Medicine* **24**: 480-493.

(Manuscript received 17 October 2008,
Revision accepted 26 November 2008)