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SUPEROXIDE DISMUTASE AND CATALASE ACTIVITIES IN THE DIGESTIVE GLAND AND GILLS OF THE FRESHWATER BIVALVE *UNIO PICTORUM* FROM THE SAVA RIVER

SLAVICA S. BORKOVIĆ-MITIĆ, TIJANA B. KOVAČEVIĆ, BRANKA R. PERENDIJA, SVETLANA G. DESPOTOVIĆ, JELENA P. GAVRIĆ, S. Z. PAVLOVIĆ and ZORICA S. SAIČIĆ*

Department of Physiology, Institute for Biological Research "Siniša Stanković", University of Belgrade, 11060 Belgrade, Serbia

Abstract – We investigated the potential use of the antioxidant defense enzymes in freshwater mussel (*Unio pictorum*) as biomarkers of oxidative stress. The enzymatic activities of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6), total protein concentration in addition to protein and SOD electrophoretic profiles were examined in the digestive gland and gills of the freshwater bivalve *Unio pictorum* at two localities on the River Sava. The differences between SOD and CAT activities in examined tissues of freshwater bivalve *Unio pictorum* reflect dissimilar metabolic and antioxidative activities and this can be the result of both tissue or locality specificities and diverse ecophysiological influences on the organism.

Key words: Superoxide dismutase, catalase, biomonitoring, *Unio pictorum*, digestive gland, gills, Sava River

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INTRODUCTION

Under normal physiological condition, animals maintain a balance between generation and neutralization of reactive oxygen species (ROS). However when organisms are subjected to xenobiotic compounds, the rate of production of ROS, such as superoxide anion radicals ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\bullet}OH$) and peroxyl radicals (ROO^-) exceeds their scavenging capacity (Halliwell and Gutteridge, 2007). All organisms have their own cellular antioxidative defense system (ADS), with both enzymatic as well as non-enzymatic components. An enzymatic pathway consists of superoxide dismutase - SOD, catalase - CAT and glutathione peroxidase - GSH-Px. SOD catalyzes the dismutation of O^{2-} molecules to H_2O_2 which is reduced to water and molecular oxygen by CAT, or is neutralized by GSH-Px which catalyzes the reduction of H_2O_2 to water and organic peroxides to alcohols using glutathione as a source of a reducing equivalent. The enzyme glutathione reductase (GR) regenerates GSH from oxidized glutathione (GSSG) which is a scavenger of ROS, as well as a substrate for other enzymes. Glutathione-S-transferase (GST) conjugates xenobiotics with GSH for excretion. Some of these parameters could serve as stress indicators in animals exposed to environmental contaminants. Antioxidant defense enzymes (ADS) play a crucial role in maintaining cell homeostasis. ADS may be induced after exposure to pollutants, this response reflecting an adaptation of the species to their environment. This system may also be inhibited, which may lead to antioxidant-mediated toxicities (Winston and Di Giulio, 1991; Doyotte et al., 1997; Cossu et al., 1997).

Biomarkers are defined as suborganismic changes occurring at cellular, biochemical, molec-

ular, or physiological levels, which can be measured in the cells, body fluids, tissues or organs within an organism and that may be indicative of xenobiotic exposure and/or effect. One of the key functions of biomarkers is to provide early warning signals of significant biological effects and it is generally believed that suborganismic(molecular, biochemical and physiological) responses precede those that occur at higher levels of biological organization such as population, community or ecosystem (Lam, 2009; Vidal-Linán et al., 2010). The antioxidative defense enzymes and non-enzymatic components of ADS have been proposed as biomarkers of contaminant mediated oxidative stress in a variety of marine and freshwater organisms (Saičić et al., 1993; Borković et al., 2005; Kovačević et al., 2006; Šaponjić et al., 2006; Despotović et al., 2007; Borković et al., 2008; Kovačević et al., 2008). Our previous reports have also considered antioxidant defense enzymes as biomarkers for oxidative stress in marine fish (Pavlović et al., 2004; Pavlović et al., 2008) and freshwater mussels (Perendija et al., 2007 a, b).

Freshwater mussels are an ecologically important fauna because they are used as sensitive biomarkers of aquatic ecosystems pollution. Hence, mussels such as *Unio pictorum* fulfill the requirements which make them useful bioindicators of chemical pollution: they have a wide geographical distribution, are easy to collect, are sessile filter-feeding organisms which may be exposed to large amounts of chemical pollutants, are capable of accumulate and tolerate high concentrations of many organic and inorganic pollutants in their tissues (Niyogi et al., 2001; Campanella et al., 2005).

SOD and CAT are two very important enzymes of the ADS in freshwater organisms. Many studies have shown positive correlations between levels of antioxidant defenses and the influence of environmental conditions (Orbea et al., 2002). The digestive gland and gills were selected according to their function in the regulation of overall body metabolism (digestive gland) and oxygen metabolism (gills).

The aim of our study was to determine and compare the physiological responses of superoxide dismutase (SOD, EC 1.15.1.1) and catalase (CAT, EC 1.11.1.6) in the digestive gland and gills of freshwater bivalve *Unio pictorum* from the River Sava, at two localities (Jamena and Šabac) that are characterized by different environmental conditions.

MATERIALS AND METHODS

Locality description and sample collection

The study is based on material collected in August 2006. The research was carried out at two sampling localities of the Sava River (Fig. 1). The freshwater mussels were collected by diving. The diver sampled all the specimens from a chosen quadrant and brought them to shore for identification (Paunović et al., 2008). The coordinates of the sampling localities were measured by GPS ("Garmin Etrex") and charted using ArcView software (map 1:300,000, system WGS_1984).

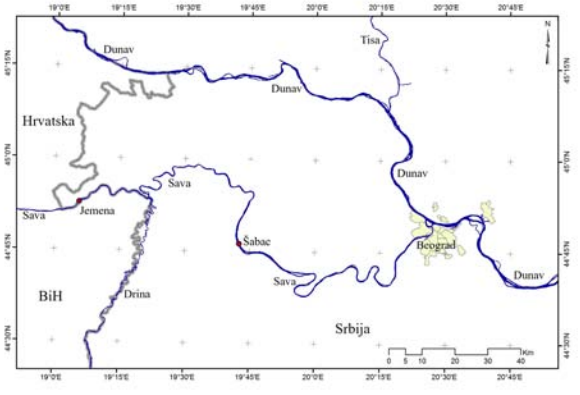

Fig. 1. Sampling localities: (1) Jamena and (2) Šabac.

Specimens of freshwater mussels *Unio pictorum* (n=10) were collected at two localities of the River Sava: Jamena (44°52'41.6" N and 19°05'21.0" E), and Šabac (44°46'17.2" N and 19°42'16.1" E). All specimens were sexually mature with a shell length about 11.62 cm (Fig. 2). After collection, the tissue samples (digestive gland and gills) were immediately dissect-

Fig. 2. Freshwater bivalve *Unio pictorum.*

ed on ice and then frozen in liquid nitrogen before storage at -80°C.

Tissue processing

The tissues were minced and homogenized in 5 volumes (Lionetto et al., 2003) of 25 mmol/L sucrose containing 10 mmol/l Tris-HCl, pH 7.5 at 4°C using an IKA-Werk Ultra-Turrax homogenizer (Janke and Kunkel, Staufen, Germany), (Rossi et al., 1983). The homogenates were sonicated for 30 s at 10 kHz on ice to release enzymes (Takada *et al.*, 1982) and then centrifuged in a Beckman ultracentrifuge (for 90 min at 85000 x g and 4°C). The resulting supernatants were used for further biochemical analyses.

Biochemical analyses

The activity of superoxide dismutase (SOD) was measured in triplicate for each mussel using a Shimadzu UV-160 spectrophotometer and a temperature controlled cuvette holder.

SOD activity was assayed by the epinephrine method (Misra and Fridovich, 1972). One unit of SOD activity was defined as the amount of protein causing 50% inhibition of the autooxidation of adrenaline at 26°C (Petrović et al., 1982). CAT activity was determined by the method of hydrogen peroxide consumption measured spectrophotometrically at 240 nm according to Claiborne (1984). The activity of both enzymes was expressed as specific (U/mg of protein) and as total (U/g wet mass) as described previously by De Quiroga et al. (1988).

Total protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as a reference and expressed in mg/g wet mass. Protein electrophoretic profiles were examined by the standard method of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), (Laemmli, 1970). SOD electrophoretic profiles were examined using NBT by the method of Mavelli et al. (1984). All chemicals were products of Sigma-Aldrich (St Louis, MO, USA).

Statistical analyses

The data are expressed as mean ± Standard Error (S.E.). The non-parametric Mann-Whitney U-test was used to seek significant differences between means. A minimum significance level of $p<0.05$ was accepted. Analytical protocols described by Darlington et al. (1973) and Dinneen and Blakesley (1973) were followed.

RESULTS

The results of the measured physico-chemical parameters at the two localities are presented in Table 1.

Table 1. Physico-chemical parameters.

Site	Jamena	Šabac
Temperature (°C)	22.21	22.76
pH	8.1	8.5
Dissolved O_2 (mg/L)	5.98	7.13
Saturation (%)	66.0	75.9

The total protein concentration in the digestive gland and gills of the freshwater mussel *U. pictorum* is shown in Table 2. The obtained results in the digestive gland demonstrate a significantly higher total protein concentration compared to the gills at both localities ($p<0.05$).

The specific and total activities of the investigated antioxidative defense enzymes in the digestive gland

Table 2. Total protein concentration (mg/g wet mass) in the digestive gland and gills of the freshwater mussel *Unio pictorum* at two localities. The data are expressed as means ± S.E. The non-parametric Mann-Whitney *U*-test was used to obtain significant differences between means. *p<0.05 represents a minimal significant level for effects of tissue.

Total protein concentration (mg/g wet mass)		
Unio pictorum		
Mean ± SE	Jamena	Šabac
Digestive gland	22.20 ± 1.13	21.76 ± 0.81
Gills	16.93 ± 0.61*	18.49 ± 0.65*

and the gills of *U. pictorum* from the River Sava are illustrated in Figs. 3A and 3B and 4A and 4B.

Specific (Fig. 3A) and total (Fig. 3B) SOD activities in the digestive gland were significantly higher compared to those in the gills (p<0.05). The results of our investigations show that the specific activity of SOD (Fig. 3A) at the two localities was considerably higher in the digestive gland (p<0.05) compared to the gills. When we compared the specific and total SOD activity between the localities (Jamena and

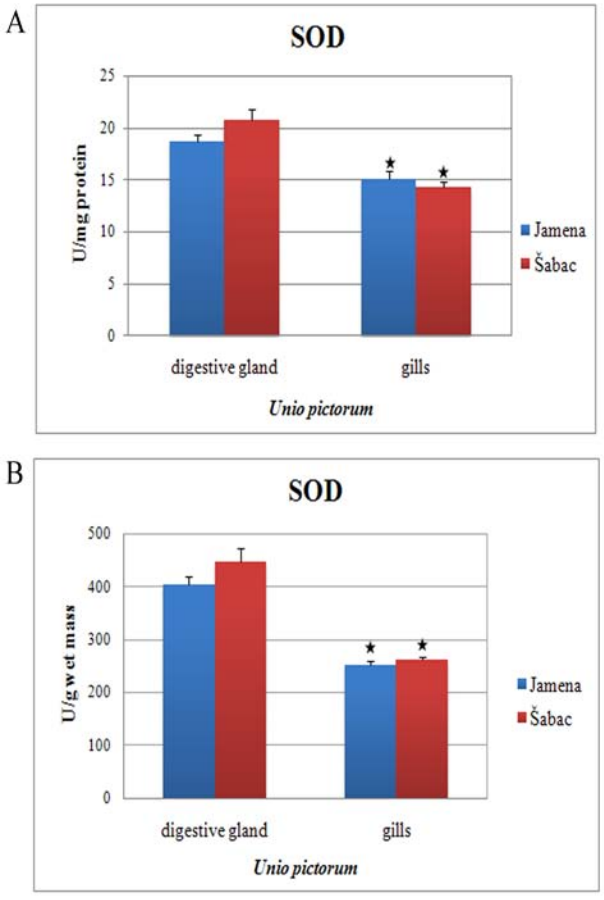

Fig. 3. Specific (A) (Units/mg protein) and total (B) (Units/g wet mass) activities of superoxide dismutase (SOD) in the digestive gland and gills of freshwater bivalve *Unio pictorum* from the two localities of the River Sava (Jamena and Šabac). The data are expressed as mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. *p<0.05 represents a minimal significant level for effects of tissue.

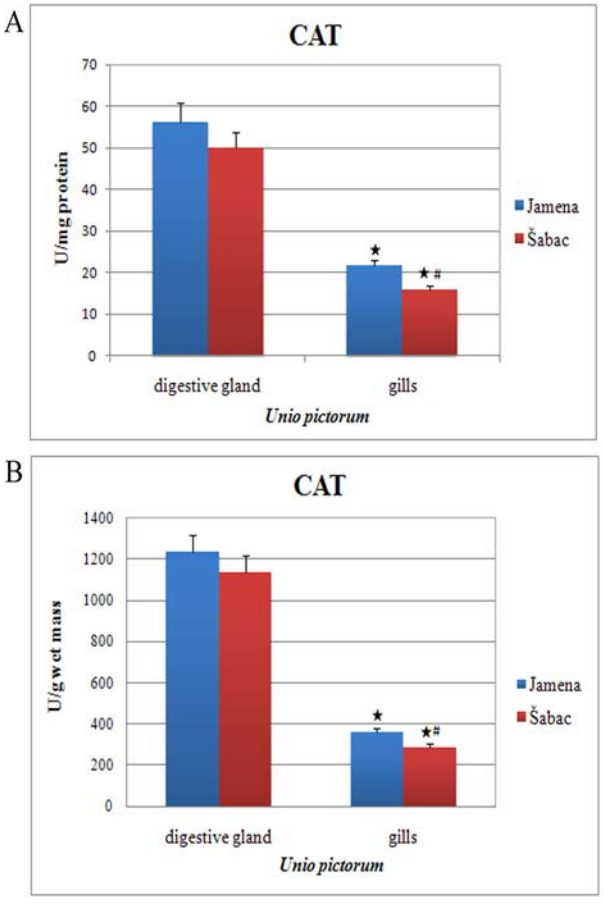

Fig. 4. Specific (A) (Units/mg protein) and total (B) (Units/g wet mass) activities of catalase (CAT) in the digestive gland and gills of freshwater bivalve *Unio pictorum* from the two localities of the River Sava (Jamena and Šabac). The data are expressed as mean ± S.E. The non-parametric Mann-Whitney *U*-test was used to seek significant differences between means. *p<0.05 represents a minimal significant level for effects of tissue; # p<0.05 represents a minimal significant level for effects of locality.

Šabac) for one selected tissue (digestive gland or gills), a statistical difference was not found. Total SOD activity (Fig. 3B) was also considerably higher in the digestive gland ($p<0.05$) compared to the gills.

A similar trend was obtained for both the specific (Fig. 4A) and the total CAT activity (Fig. 4B). The specific activity of CAT was significantly higher in the digestive gland when compared to that in the gills. A significant statistical difference was found between specific and total CAT activity in the gills on the two different localities, with higher activity in Jamena. The total CAT activity was also significantly greater in the digestive gland ($p<0.05$) compared to the gills.

Electrophoretic analysis of proteins shows characteristic protein profiles in the examined tissues of mussel *U. pictorum* from the River Sava (Fig. 5A and 5B).

SOD activity was analyzed directly on gels after native electrophoresis using the NBT method (Figs. 6A and 6B).

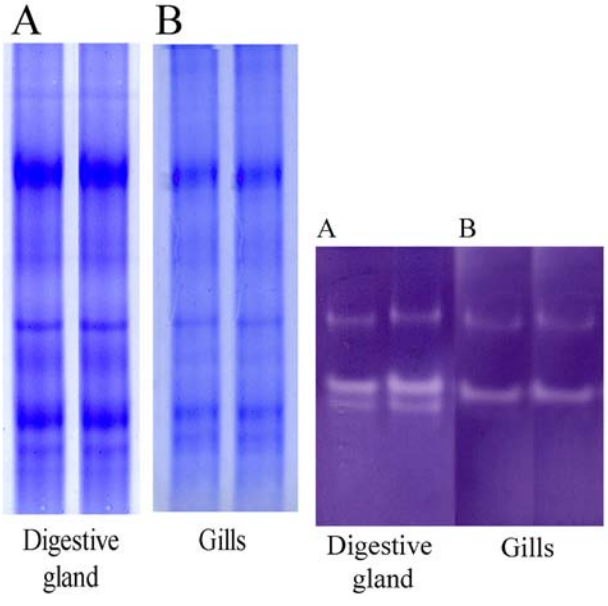

Fig. 5. SDS-PAGE analysis of proteins in the digestive gland (A) and gills (B) of freshwater bivalve *Unio pictorum*. Two samples at each investigated tissues were analyzed.

DISCUSSION

A difference in tissue expression was reported between the digestive gland and gills with a generally higher activity of investigated enzymes in digestive gland. Moreover, although the digestive gland is of particular interest because this tissue is involved in most biotransformation processes and redox-cycling generation, it showed high fluctuations in the activity levels between samplings, thus rendering interpretation of the results inconsistent, unlike those obtained from the gills. The digestive gland is the site of multiple oxidative reactions and may therefore be a site of substantial free radical generation. In the gills of freshwater mussels the specific and total SOD and CAT activities were significantly lower when compared to the digestive gland. Our results suggest that gills exhibit a low-threshold response to oxidative stress, as the organ is the first tissue to come into contact with potential water-borne contaminants. The gills intracellular metabolism must also be coordinated and primed in order to provide a first line of antioxidative defense.

Changes in temperature and food availability induce oxygen consumption and cellular oxyradical generation, which are compensated by increasing the antioxidative defense.

The differences in the protein concentration in the investigated tissues could be a consequence of various external influences, such as different food availability or the different degrees of pollution at the investigated localities. Furthermore, the differences between the total protein concentrations in the investigated tissues and the analysis of the protein electrophoretic profiles show strong tissue specificities.

Electrophoresis under native conditions allowed for the simultaneous characterization of SOD isoforms. Manduzio et al. (2003) detected three isoforms of SOD in the digestive gland extracts. Two major bands, named SOD-1 and SOD-2, were obtained at pI 4.7 and pI 4.6, respectively. A third band at pI 4.55, named SOD-3, was not systematically visualized in the tissue extracts from the mussels collected

at the reference site. It was always distinguished by very weak intensity (10% of the total SOD activity) compared to the two others. In contrast, SOD-3 was always present and more highly expressed in the digestive glands of mussels taken at the polluted site (20-40% of the total SOD activity). We also detected three SOD isoforms in the digestive gland of the mussels *U. pictorum* (Fig. 6 A). They exhibited three different isoelectric points: 4.7, 4.6 and 4.55, respectively. Manduzio et al. (2003) described the possibility of induction of this last isoform SOD-3 under experimental conditions, in particular, after exposure of mussels to contamination. If the activity of the SOD is generally considered as stable, the study of the SOD isoform pattern is new in bivalves. In this study we demonstrate the usefulness of the modifications of the SOD pattern *in situ*, for biomonitoring studies.

CAT was more active in the digestive gland, confirming the expression characteristics of this enzyme (Power and Sheehan, 1996). Because SOD is present at a lower level than CAT in the digestive gland, we hypothesize that the H_2O_2 eliminated by CAT is mainly derived from the divalent reduction of O_2, performed by various oxidases in peroxisomes. Some authors have hypothesized that the H_2O_2 dismutated by CAT could be produced by monovalent reduction, which implies the presence of SOD in these organelles (Santovito et al., 2002). It is known that SOD and GSH-Px were found in the cytosol and mitochondria. CAT was detected only in peroxisomes, whereas the presence of SOD in these organelles is controversial (Santovito et al., 2005). CAT response to toxic chemicals shows a bell-shaped trend, with an initial increase in activity due to enzyme induction, followed by a decrease in activity due to an enhanced catabolic rate and/or direct inhibition by toxic chemicals (Viarengoet al., 2007). Such trends in CAT activity can be found in mussels at polluted sites according to the levels and duration of pollutant exposure (Nasci et al., 2002; Regoli et al., 2004; Nesto et al., 2004; Pampanin et al., 2005; Tsangaris et al., 2010). Regoli et al. (2004) showed an increase in CAT activity during the first two weeks of mussel transplantation at an industrialized harbor in Italy, followed by a progressive decrease. Decreased CAT activity in mussels transplanted to polluted sites has been found in addition to a reduced capability to neutralize ROS and an increased susceptibility to oxidative stress (Pampanin et al., 2005).

In conclusion, the differences between the SOD and CAT activities in the examined tissues of the freshwater bivalve *Unio pictorum* reflect diverse metabolic and antioxidative defense activities and this can be the result of both species or tissue specificities and of various ecophysiological influences on the organism. This study supports the use of SOD and CAT as useful biomarkers for long-term pollution monitoring of river ecosystems. We also observed a third band of electrophoretic profiles of SOD, named SOD-3, in the digestive gland of mussels. Our results suggest that variations of SOD expression patterns in *Unio pictorum* could be used as a tool for the environment monitoring.

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