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# GLUTATHIONE DEPENDENT ENZYME ACTIVITIES IN THE FOOT OF THREE FRESHWATER MUSSEL SPECIES IN THE SAVA RIVER, SERBIA

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*Abstract* – We investigated activities of glutathione peroxidase (GSH-Px), glutathione reductase (GR), and the phase II biotransformation enzyme glutathione-S-transferase (GST) in the foot of three freshwater mussel species: *Unio pictorum* (Up), *Unio tumidus* (Ut), and *Sinanodonta woodiana* (Sw) from the Sava River. Specific and total GSH-Px activity was lower in Sw than in Up and Ut. Total GR activity was higher in Up than in Sw. Specific GST activity was higher in Up than in Ut. Total GST activity was higher in Up than in Ut and Sw. Electrophoretic analysis of proteins shows species specifities between the investigated mussel species. Our study represents the first comprehensive report of the investigated glutathione-dependent enzyme activities in the foot of three freshwater mussel species from the Sava River, Serbia.

*Key words:* Glutathione peroxidase, glutathione reductase, glutathione-S-transferase, Sava River, *Sinanodonta woodiana, Unio pictorum, Unio tumidus.*

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## INTRODUCTION

Activity of the antioxidant defense system can be increased or inhibited under chemical stress and antioxidant parameters therefore represent biomarkers of interest (D o y o t t e et al., 1997). Studies on aquatic species have been carried out mainly on marine species (L i v i n g s t o n e et al., 1990; D i G i u l i o et al., 1993) and little is known about the response of the antioxidant defense system to pollutants in freshwater species. Bivalve mollusks are commonly used as bioindicators in the assessment of environmental quality because they are widely distributed, live in direct contact with the substrate, and have a high capacity for bioaccumulation. A number of antioxidant defense mechanisms are present in bivalve mollusks, including low-molecular-weight compounds and specially adapted enzymes (W i n s t o n, 1991). Use of the biotransformation enzymes involved in cellular detoxification as biomarkers of exposure to xenobiotics has been intensively studied in mollusks (P e t e r s et al., 1999; M o r e i r a et al., 2001).

Glutathione peroxidase (GSH-Px), glutathione reductase (GR), and glutathione-S-transferase (GST) are glutathione-dependent enzymes because they use glutathione (GSH) as a cofactor. The first two enzymes are antioxidant defense enzymes, while GST is a phase II biotransformation enzyme. The ubiquitous tripeptide GSH (L-γ-glutamyl-cysteinyl-glycine), the most abundant soluble cellular thiol, is involved in processes essential for synthesis and degradation of proteins, formation of deoxyribonucleotides, regulation of enzymes, and protection of cells against reactive oxygen species (M e i s t e r and A n d e r s o n, 1983). Glutathione exists in two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). The former is present usually in high concentrations in tissues and is one of the most important endogenous antioxidants. On the other hand, GSSG is toxic and can be used as an indication of oxidative stress (P a r r i s and K i d d, 1997).

Glutathione peroxidase binds GSH with high affinity and oxidizes it to GSSG. This enzyme can detoxify hydrogen peroxide and organic hydroperoxides which are formed during tissue oxidative stress. Glutathione reductase reduces GSSG and is thus at the base of the regeneration of GSH necessary to the operation of GSH-Px, GST, and many other enzymes of the cell (Winston and Di Giulio, 1991). The phase II biotransformation enzyme GST catalyzes the conjugation of GSH to a wide variety of xenobiotics with an electrophilic site, yielding xenobiotics more water soluble and facilitating their excretion (Mannervik and Danielson, 1988).

The aim of our study was to determine activites of the glutathione dependent enzymes GSH-Px (EC 1.11.1.9), GR (EC 1.6.4.2), and the phase II biotransformation enzyme GST (EC 2.5.1.18) in the foot of three freshwater mussel species: *Unio pictorum* (Painter's mussel), *Unio tumidus* (swollen river mussel), and *Sinanodonta woodiana* (Chinese pond mussel) from the Sava River near the city of Šabac. The first two species are native to Serbian freshwaters, but *S. woodiana* is an invasive species and originates from Eastern Asia (Paunović et al., 2006). We also measured the concentration of total proteins and considered the gross pattern of mussel proteins using electrophoresis.

## MATERIALS AND METHODS

### *Sample collection and preparation*

Specimens of freshwater mussels (n=30, 10 of each species) were collected from the Sava River near the city of Šabac (44° 46' 17. 2'' N and 19° 42' 16. 1'' E at an altitude of 70 m above sealevel) (Fig. 1) in August of 2006. The foot of each mussel species was dissected, put it in liquid nitrogen, and then stored at -80°C until used for further biochemical analysis. 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 and then were centrifuged at 4°C at 100000 x *g* for 90 min (Takada et al. 1982). The resulting supernatants were used for biochemical analyses.

### *Biochemical analyses*

Activities of glutathione-dependent enzymes were measured simultaneously in triplicate for each mussel using a Shimadzu UV-160 spectrophotometer and a temperature-controlled cuvette holder. The activity of GSH-Px was determined following oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) as a substrate with t-butyl hydroperoxide (Tamura et al., 1982) and expresed in nmol of NADPH/min/mg of protein, as well in nmol of NADPH/min/g of wet mass. Glutathione reductase activity was measured as described by Glatzle et al. (1974) and expresed in nmol of NADPH/min/mg of protein and in nmol of NADPH/min/g of wet mass. Activity of GST toward 1-chloro-2,4-dinitrobenzene (CDNB) was assayed by the method of Habig et al. (1974) and expresed as nmol of GSH/min/mg of protein and as nmol of GSH/min/g of wet mass. The activities of glutathione-dependent enzymes were expressed as specific (U/mg of protein) and total (U/g of wet mass) activities as described by Barja De Quiroga et al. (1988). Total protein concentration in the supernatant was determined according to the method of Lowry et al. (1951) and expressed in


Fig. 1. Site of specimen collection from the Sava River near the city of Šabac (44° 46' 17. 2'' N and 19° 42' 16. 1'' E at an altitude 70 m above sealevel).

mg/mL. Protein electrophoretic profiles were examined by the standard method of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), (L a e m m l i, 1970). All chemicals were products of Sigma-Aldrich (St. Louis, MO, USA).

*Statistical analyses*

Data are given as the mean ± SE (standard error). Statistical significance between the species was analyzed using the unpaired Student t-test considering at a level of $p<0.05$ to be significant (H o e l, 1966).

## RESULTS

Total protein concentration of the mussels is shown in Table 1. The obtained results demonstrate significantly lower total protein concentration in *S. woodiana* compared to the other two species, *U. pictorum* and *U. tumidus* ($p<0.005$). Data on glutathione dependent antioxidant enzyme activities are given in Figs. 2-4. Specific and total GSH-Px activity was significantly lower in *S. woodiana* than in *U. pictorum* and *U. tumidus* ($p<0.005$) (Figs. 2A and 2B). As presented in Fig. 3B, total GR activity was considerably higher in *U. pictorum* than in *S. woodiana* ($p<0.005$). Specific GST activity was significantly higher in *U. pictorum* than in *U. tumidus* ($p<0.005$) (Fig. 4A). Also, total GST activity was considerably higher in *U. pictorum* than in *U. tumidus* and *S. woodiana* ($p<0.005$) (Fig. 4B). Electrophoretic analysis of proteins shows species specifities between the investigated freshwater mussel species (Fig. 5).

## DISCUSSION

Different animals in aquatic ecosystems have developed various ways of protection from changing environmental conditions and pollution (A d a m s et al., 2000). Useful as indicators in biomonitoring investigations are the activities of antioxidant defense enzymes and biotransformation enzymes. Activity of the antioxidant defense system can be increased or inhibited under chemical stress. These two kinds of response depend on the intensity and duration of the stress applied and on susceptibility of the exposed living species. Induction of the antioxidant defense system can be considered an adaptation of species to their environment, whereas inhibition may lead to antioxidant-mediated toxicities (W i n s t o n and D i G i u l i o, 1991). The responses in mussels make

Table 1. Total protein concentration (mg/mL) in the foot of *Unio pictorum* (Up), *Unio tumidus* (Ut), and *Sinanodonta woodiana* (Sw).

| | *Unio pictorum* | *Unio tumidus* | *Sinanodonta woodiana* | Statistical significance |
|---|---|---|---|---|
| Protein concentration | 6.70 ± 0.11 | 6.69 ± 0.21 | 5.65 ± 0.17 | Up vs Sw: $p<0.005$<br>Ut vs Sw: $p<0.005$ |

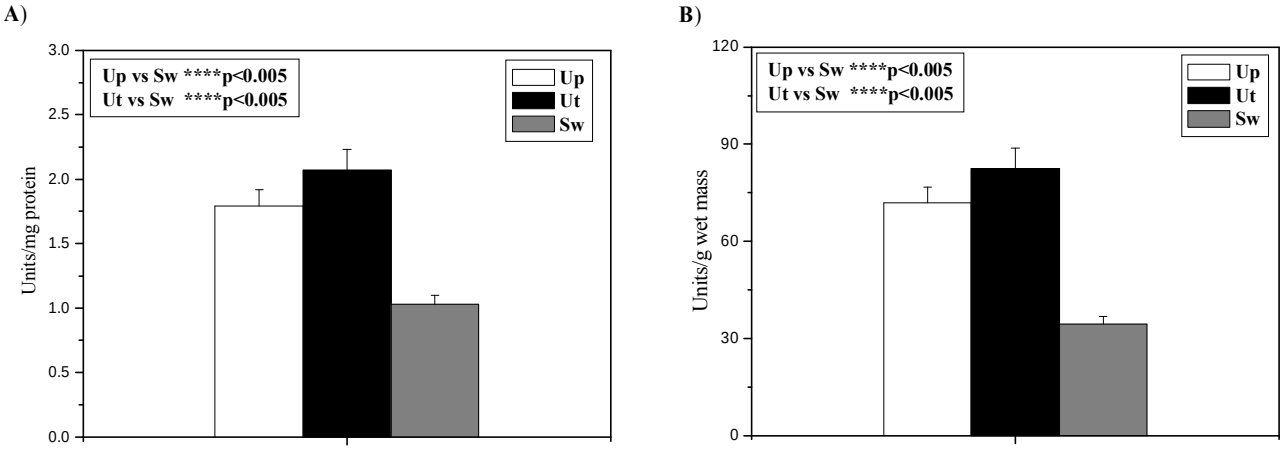



Fig. 2. Specific (A) and total (B) activities of GSH-Px in the foot of *Unio pictorum* (Up), *Unio tumidus* (Ut), and *Sinanodonta woodiana* (Sw).

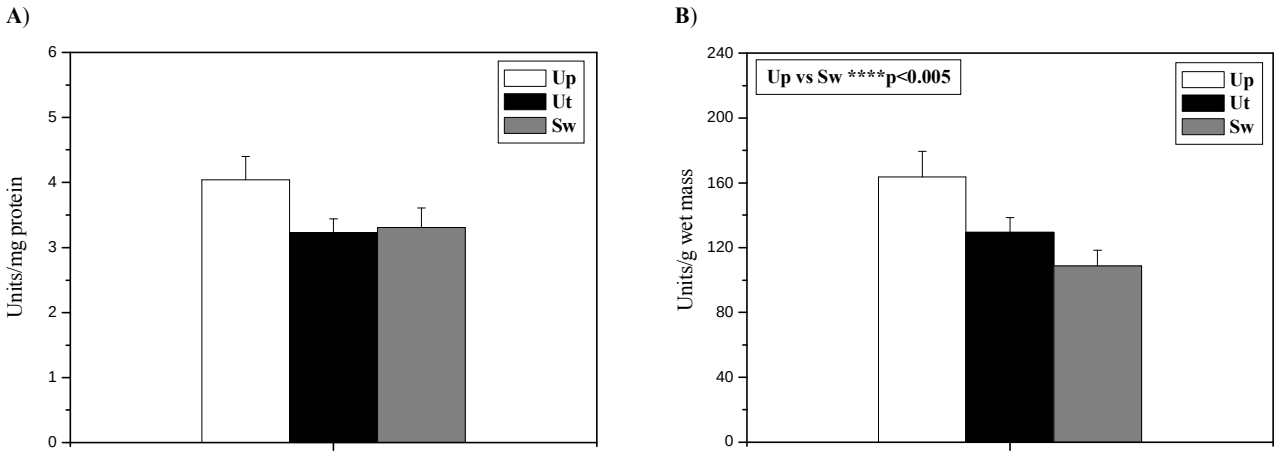



Fig. 3. Specific (A) and total (B) activities of GR in the foot of *Unio pictorum* (Up), *Unio tumidus* (Ut), and *Sinanodonta woodiana* (Sw).

them good bioindicators for environmental monitoring (L i v i n g s t o n e, 1993). A great number of biomarker studies have been performed on bivalves for the following reasons: their wide distribution, direct contact with the substrate, great tolerance to a huge variety of environmental conditions, and significant bioconcentration of environmental toxicants due to high filtration activity.

The results obtained in our study show that total and specific GSH-Px activities were significantly lower in *S. woodiana* compared to the other investigated freshwater species, *U. pictorum* and *U. tumidus*. C o s s u et al. (2000) observed the antioxidant defense system in the freshwater bivalve *U. tumidus* transplanted from a control site to four different contaminated areas and reported that the most susceptible antioxidant parameters were selenium-dependent GSH-Px (Se GSH-Px) and GR activities, which decreased. Decline of those parameters indicates that the mussels were under oxidative stress as a result of exposure to prooxidant chemicals. G é r e t et al. (2002) also detected a reduction in the levels of SeGSH-Px and total GSH-Px activities in *Ruditapes decussates* exposed to copper after one day of exposure. The enzyme SeGSH-Px is considered an efficient protective agent against lipid peroxidation (W i n s t o n and D i G i u l i o, 1991). On the other hand, in some studies where the induction of SeGSH-Px was recorded, this increase was not sufficient to prevent oxidative damage (D i G i u l i o et al., 1993). J i n g et al. (2006) maintained that GSH-Px may be potentially useful as a biomarker in biotesting of metal pollution.

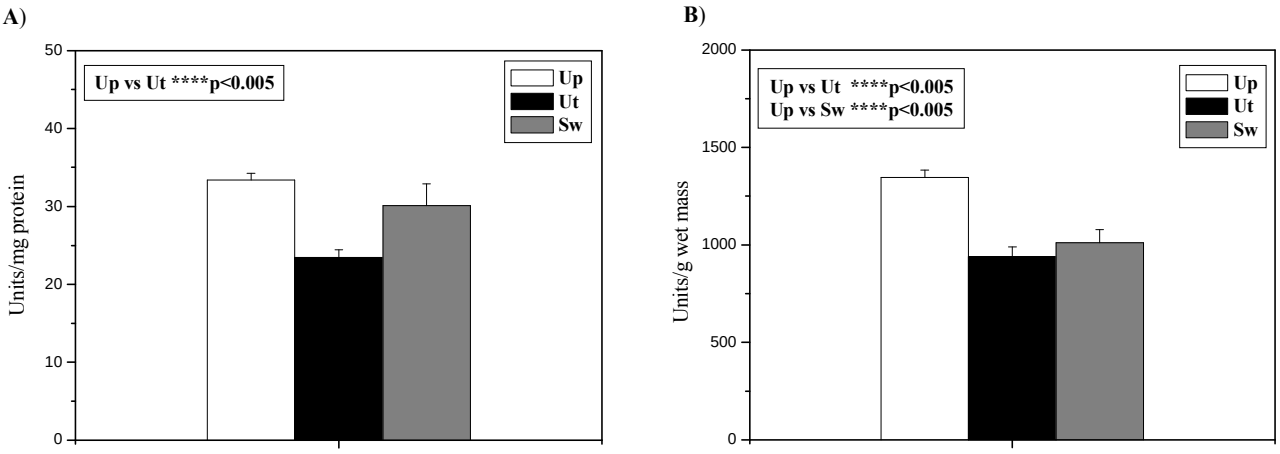
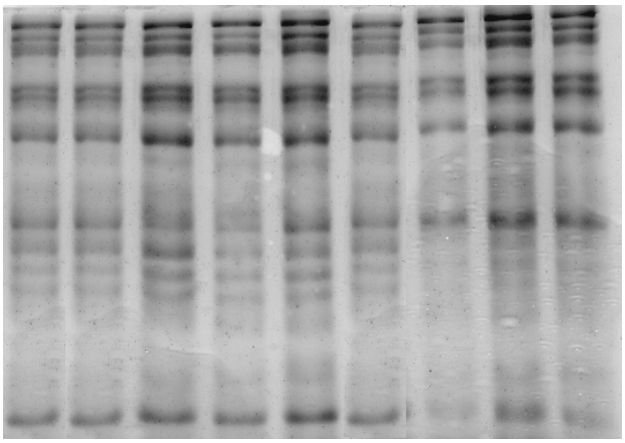



Fig. 4. Specific (A) and total (B) activities of phase II biotransformation enzyme GST in the foot of *Unio pictorum* (Up), *Unio tumidus* (Ut), and *Sinanodonta woodiana* (Sw).



**Up1 Up2 Up3 Ut1 Ut2 Ut3 Sw1 Sw2 Sw3**

Fig. 5. SDS-Page analysis of protein profile in the foot of *Unio pictorum* (Up), *Unio tumidus* (Ut), and *Sinanodonta woodiana* (Sw). Three samples of each investigated mussel species were analyzed.

In our experiments, we found that total GR activity was higher in *U. pictorum* than in *S. woodiana.* Glutathione reductase has a crucial role in cellular antioxidant protection because of its ability to regenerate GSH. Previous reports indicated that GR activity decreased during exposure of the bivalve *Anadara granosa* to mercury (Patel et al., 1990). Doyotte et al. (1997) measured GR activity in the gills and digestive gland of *U. tumidus* after exposure to copper and/or thiram and observed a reduction of its activity. The same authors also found that GR activity is a more sensitive parameter than activity of SeGSH-Px.

Specific GST activity was significantly higher in *U. pictorum* than in *U. tumidus*, while total GST activity was considerably higher in *U. pictorum* than in *U. tumidus* and *S. woodiana*. As a phase II biotransformation enzyme, GST has been used as a biomarker of organic industrial effluents (Sheehan et al., 1995). In addition, GST has been used as a biomarker of exposure to anthropogenic organics (Fitzpatrick et al., 1997). Di Giulio et al. (1993) reported induction of some GST isoenzymes by substrates such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenols (PCBs). Lenartova et al. (1997) also reported induction of GST, but after exposure to metals. This enzyme is the most sensitive biomarker of the influence of environmental pollution on the organism and its activity has been shown to increase in the whole organism or particular organs (gills, digestive gland) as a function of the xenobiotic concentration (Stien et al., 1998). Some authors found that the activity of GST in mussels was significantly higher in winter, which is in accordance with higher sensitivity to oxidative stress during this period (Van der Oost et al., 2003; Borković et al., 2005).

Total protein concentration of the mussels was significantly lower in *S. woodiana* than in *U. pictorum* and *U. tumidus*. Electrophoretic analysis of proteins also shows species specifities between the investigated freshwater mussel species.

In conclusion, our present study represents the first comprehensive report on activities of the investigated glutathione-dependent enzymes (GSH-Px, GR, and the phase II biotransformation enzyme GST) in the foot of three freshwater mussel species (*Unio pictorum, Unio tumidus* and *Sinanodonta woodiana*) from the Sava River. It is important to note that the presented experiments were the first ones ever performed on the non-indigenous species *Sinanodonta woodiana* from the Serbian part of the Sava River. The parameters used in our work can be useful biomarkers for estimation of the effects of environmental contamination on freshwater invertebrates.

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# АКТИВНОСТ ГЛУТАТИОН ЗАВИСНИХ ЕНЗИМА У СТОПАЛУ ТРИ ВРСТЕ СЛАТКОВОДНИХ ШКОЉКИ ИЗ РЕКЕ САВЕ, СРБИЈА

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Одређивали смо активност глутатион-пероксидазе (GSH-Px), глутатион-редуктазе (GR) и ензима фазе II биотрансформације глутатион-С-трансферазе (GST) у стопалу три врсте слатководних шкољки: *Unio pictorum* (Up), *Unio tumidus* (Ut) и *Sinanodonta woodiana* (Sw) из реке Саве. Специфична и укупна активност GSH-Px је била мања код Sw у односу на Up и Ut. Укупна активност GR је била већа код Up у поређењу са Sw. Специфична активност GST је била већа код Up у односу на Ut. Укупна активност GST је била већа код Up у поређењу са Ut и Sw. Електрофоретска анализа протеина показује специфичност за врсту код испитиваних шкољки. Наша студија представља први обиман извештај о активности глутатион зависних ензима у стопалима три врсте слатководних шкољака из реке Саве.