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GLUTATHIONE AS A SUITABLE BIOMARKER IN HEPATOPANCREAS, GILLS AND MUSCLE OF THREE FRESHWATER CRAYFISH SPECIES

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Abstract — We determined the contents of total glutathione (tGSH), reduced glutathione (GSH), and oxidized glutathione (GSSG) and values of the glutathione redox index (GSH RI) in hepatopancreas, gills, and muscle of three freshwater crayfish species: noble crayfish (*Astacus astacus*) from the Southern Morava River, stone crayfish (*Austropotamobius torrentium*) from the Krajkovačka River, and spinycheek crayfish (*Orconectes limosus*) from the Danube River. The obtained data show strong tissue and species specifity of investigated parameters: tGSH, GSH, GSSG, and GSH RI in the hepatopancreas, gills, and muscle of the indicated crayfish species. Our work represents the first study of its kind and showed that the investigated parameters can be considered suitable biomarkers of the cellular glutathione redox status in of freshwater crayfish species.

Key words: Crayfish, gills, glutathione, glutathione redox index, hepatopancreas, muscle.

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INTRODUCTION

Glutathione (GSH) is the most important water-soluble low-molecular antioxidant. It is widely distributed in all living organisms and is found largely inside cells, where it reaches relatively high concentrations (0.1–10 mM) (Deneke and Fanburg, 1989). In contrast, values of GSH content in the plasma are usually in the low micromolar range, but they may vary extensively. The given antioxidant accounts for 90% of intracellular nonprotein thiols and it is therefore the most important intracellular reducing agent. GSH is a three-peptide (L-γ-glutamyl-L-cysteinyl-glycine) present in the free or bound form in the cytosol, nucleus, and mitochondria (Sen et al., 1992). There are two forms: reduced glutathione (GSH) and its oxidized dimeric form, glutathione disulfide (GSSG). Mitochondria contain 15 to 20% of total cellular GSH, which is also present in the nucleus and endoplasmic reticulum (Bribiva et al., 1993; Voehringer et al., 1998).

Numerous physiological functions have been attributed to GSH: 1) scavenging of hydrogen peroxide and other peroxides and free radicals; 2) preservation of SH groups in a reduced state in proteins, enzymes, and some other molecules; 3) detoxification of foreign compounds by their conjugation with GSH; 4) acting as a co-enzyme for some enzymes (e.g., glyoxalase); 5) translocation of amino acids across cell membranes; and 6) catalysis of disulfide exchange reactions (Meister, 1985). In general, GSH plays a key role in cellular defence and serves as a reservoir for the amino acid cysteine. GSH depletion leads to cell death and has been documented in many degenerative conditions. Mitochondrial GSH depletion may be the ultimate factor determining vulnerability to oxidant attack.

Cellular GSH contents reflect a steady-state balance between its synthesis and loss. Generation of GSH occurs as a result of *de novo* synthesis from particular amino acids, as well as due to glutathione reductase (GR)-mediated regeneration of GSH from GSSG. In some conditions (such as stress or the presence of xenobiotics), GSH is transformed into GSSG. An elevated content of GSSG can be regarded as oxidative stress marker, while the GSH/GSSG

redox couple can serve as an available index of redox state (Slivka et al., 1987).

Crayfish species exist in diverse conditions. For example, noble crayfish *(Astacus astacus)* lives in flatland rivers (such as the Southern Morava) where water is uncontaminated. Stone crayfish *(Austropotamobius torrentium)* colonizes clean mountain streams (like the Krajkovačka River) with a fast current. A third species, spinycheek crayfish (*Orconectes limosus)* inhabits the Danube River, which is much more contaminated, and we detected it for the first time in the Serbian part of the Danube (Pavlović et al., 2006). The gills and hepatopancreas were chosen for the present work because gills are the respiratory organs and exposed to ambient oxygen, while the hepatopancreas is responsible for regulation of overall body metabolism (Muriana et al., 1993). Also, the hepatopancreas is the organ most involved with the detoxification of xenobiotics. Muscle is important because of its role as a consumer.

The aim of this study was to investigate the contents of total glutathione (tGSH), reduced glutathione (GSH), and oxidized glutathione (GSSG) in the hepatopancreas, gills, and muscle of three freshwater crayfish species: noble crayfish (*Astacus astacus*), stone crayfish (*Austropotamobius torrentium*), and spinycheek crayfish (*Orconectes limosus*). We also evaluated the glutathione redox index (GSH RI), which is a comprehensive marker of the mutual relationship between components of the GSH system (Benzi et al., 1988).

MATERIAL AND METHODS

Site description, sample collection, and preparation of specimens

Three freshwater crayfish species were collected: noble crayfish (*Astacus astacus*) from the Southern Morava River (42°30'44" N, 21°53'42.1" E); stone crayfish (*Austropotamobius torrentium*) from the Krajkovačka River (43°20'48.3" N, 21°38'23.7" E); and spinycheek crayfish (*Orconectes limosus*) from Danube River (44°41'31.6" N, 20°57'38.5" E). The sites are indicated in Fig. 1. The specimens (n=30, 10 of each of the all three freshwater crayfish species)

Fig. 1. Geographical position of the Southern Morava, Krajkovačka, and Danube Rivers.

were collected (specimens of both sexes with similar dimensions and similar weights were taken in order to avoid age differences) in summer (July and August, 2004) using deep nets or by hand from their natural habitats. Water temperature was 18°C in the Southern Morava River, 14°C in the Krajkovačka River, and 19°C in the Danube River. The hepatopancreas, gills, and muscle of each crayfish species were immediately dissected and frozen in liquid nitrogen (at the site of collection) and then stored at -80°C before further biochemical analyses. All chemicals were from Sigma (St. Louis, MO, U. S. A.).

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 with an Ultra-Turrax homogenizer (Janke and Kunkel, IKA-Werk, Staufen, Germany) (Rossi et al., 1983). The homogenates were sonicated for 30 s at 10 kHz on ice (Takada et al., 1982) and then used for determination of the contents of total GSH, reduced GSH, and oxidized glutathione.

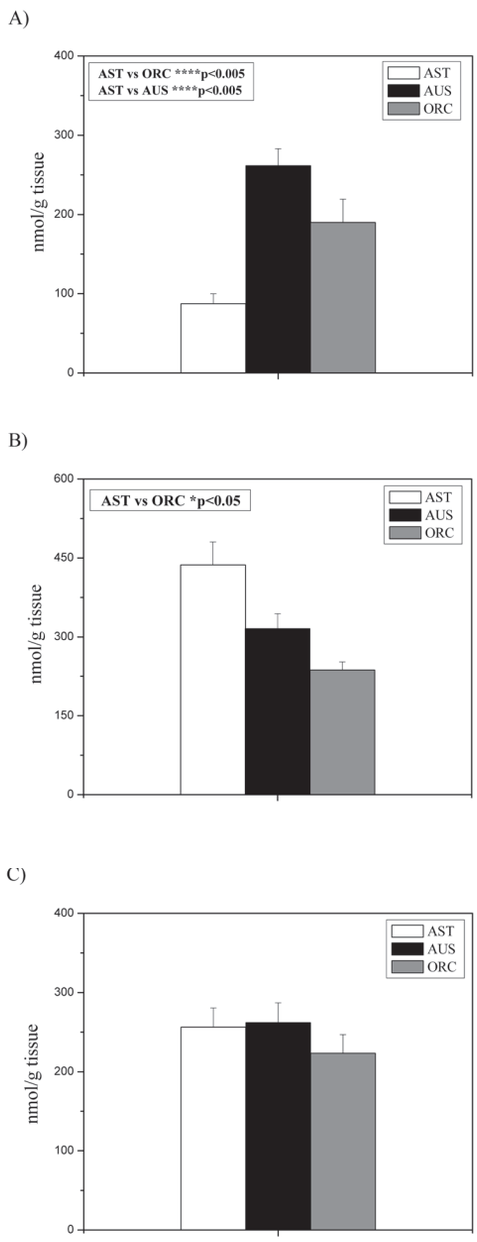


Fig. 2. Content of total glutathione (tGSH) expressed as nmol/g tissue in hepatopancreas (A), gills (B), and muscle (C) of *Astacus astacus* (AST), *Austropotamobius torrentium* (AUS), and *Orconectes limosus* (ORC).

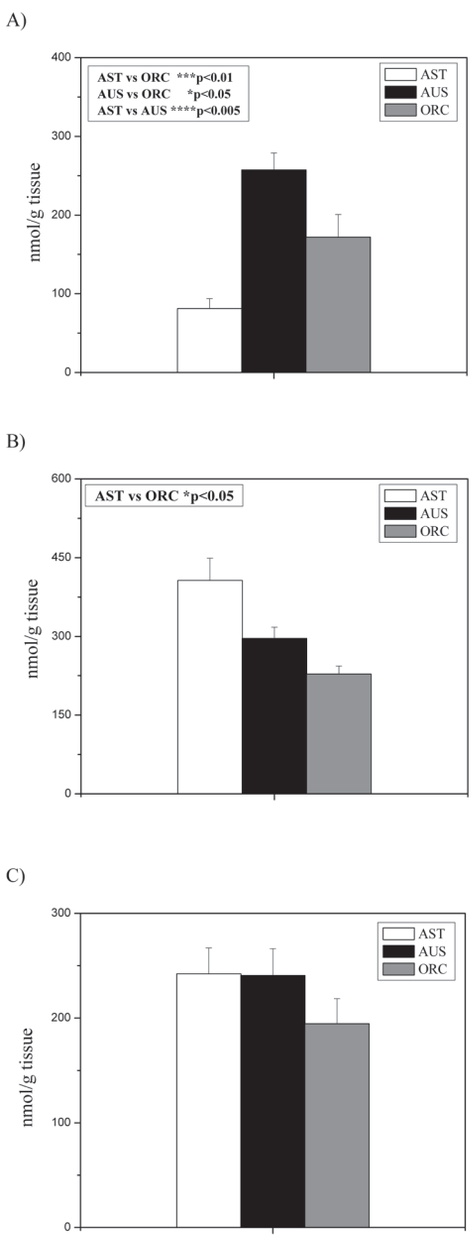


Fig. 3. Content of reduced glutathione (GSH) expressed as nmol/g tissue in hepatopancreas (A), gills (B), and muscle (C) of *Astacus astacus* (AST), *Austropotamobius torrentium* (AUS), and *Orconectes limosus* (ORC)..

Biochemical analyses

Sonicates were mixed with 10% sulfosalicylic acid (SSA) and then centrifuged at 5000 rpm for 10 min, and the resulting supernatants were stored at -80°C. The method is based on sequential oxidation of GSH by 5, 5′-dithiobis 2-nitrobenzoic acid (DTNB) and reduction by NADPH in the presence of GR (Griffith, 1980). The content of total GSH was measured in triplicate using a Shimadzu UV-

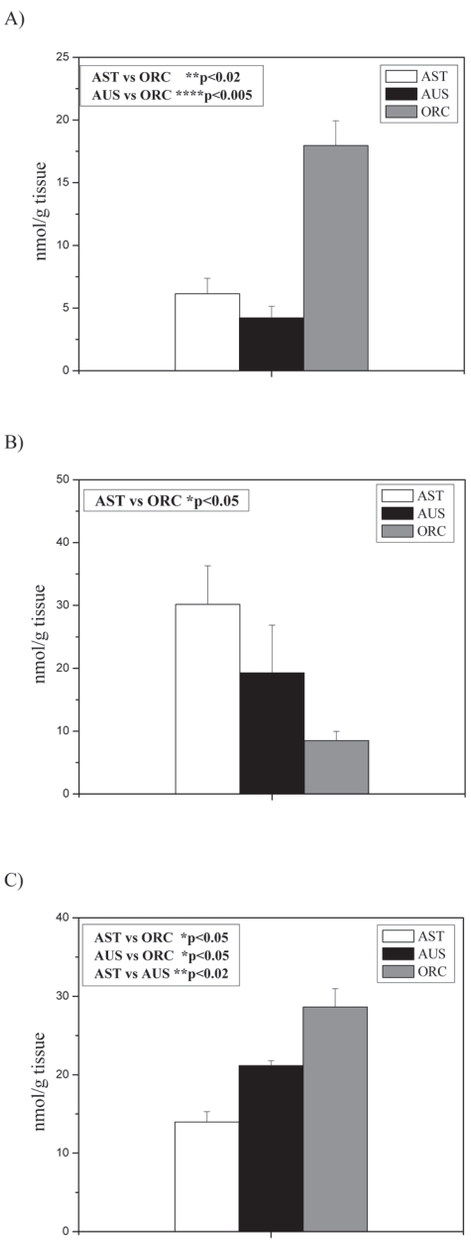
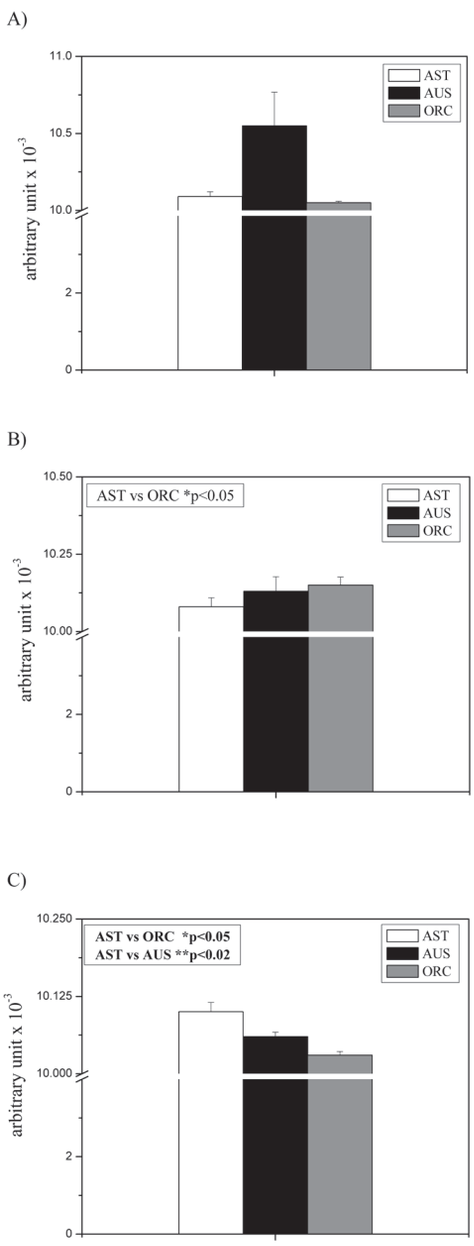


Fig. 4. Content of oxidized glutathione (GSSG) expressed as nmol/g tissue in hepatopancreas (A), gills (B), and muscle (C) of *Astacus astacus* (AST), *Austropotamobius torrentium* (AUS), and *Orconectes limosus* (ORC).



Figs. 5. Glutathione redox index (GSH RI) expressed as arbitary units in hepatopancreas (A), gills (B), and muscle (C) of *Astacus astacus* (AST), *Austropotamobius torrentium* (AUS), and *Orconectes limosus* (ORC).

160 spectrophotometer and a temperature-controlled cuvette holder. The GSSG content was obtained after GSH reaction with vinyl pyridine. The content of reduced GSH was obtained by subtracting oxidized GSH from total GSH. The contents of tGSH, GSH, and GSSG were expressed as nmol/g tissue. The glutathione redox index (GSH RI) was calculated by formula and was expressed in arbitrary units (B e n z i et al., 1988):

GSH RI = ([GSH] + 2 [GSSG]) / (2 [GSSG] x 100)

Statistical analysis

The data are expressed as means ± standard error (S.E.). Statistical differences between the species were analyzed using the unpaired Student *t*-test, a level of $p<0.05$ being considered significant (Hoel, 1966).

RESULTS

The tGSH content in the hepatopancreas (Fig. 2A) of *A. astacus* was significantly lower than in that of *A. torrentium* and *O. limosus* ($p<0.005$). In gills (Fig. 2B) of *A. astacus*, tGSH content was significantly higher than in gills of *O. limosus* ($p<0.05$). There were no significant differences between content of tGSH in muscle of the investigated crayfish species (Fig. 2C).

Content of GSH was significantly increased in the hepatopancreas of *A. torrentium* in comparison with *A. astacus* ($p<0.005$) and *O. limosus* ($p<0.05$) (Fig. 3A). We also found significant differences of GSH content between *A. astacus* and *O. limosus* ($p<0.01$). In gills of *A. astacus*, content of GSH was significantly increased (Fig. 3B) in comparison with *O. limosus* ($p<0.05$). No statistically significant differences of GSH content in muscle were found between the investigated crayfish species (Fig. 3C). The GSSG content in the hepatopancreas (Fig. 4A) of *O. limosus* was significantly higher than in that of *A. torrentium* ($p<0.005$) and *A. astacus* ($p<0.02$). In gills of *A. astacus*, GSSG content was significantly higher (Fig. 4B) than in gills of *O. limosus* ($p<0.05$). Content of GSSG was significantly increased in muscle of *O. limosus* in comparison with *A. astacus* and *A. torrentium* ($p<0.05$). We also found statistical significant differences of GSSG content between *A. astacus* and *A. torrentium* ($p<0.02$) (Fig. 4C).

Values of the glutathione redox index in the hepatopancreas were equal in the three freshwater crayfish species, so there were no significant differences. In gills of *O. limosus,* GSH RI was significantly higher than the GSH RI in gills of *A. astacus* ($p<0.05$). GSH RI was significantly increased in muscle of *A. astacus* in relation to its values in muscle of *A. torrentium* ($p<0.02$) and *O. limosus* ($p<0.05$) (Figs 5A, 5B, and 5C).

DISCUSSION

The habitats of crustaceans are characterized by unstable physico-chemical properties, so they are exposed to daily and seasonal fluctuations in temperature and concentration of dissolved oxygen (Filho, 1996). Crustaceans are poikilothermic organisms, so they continuously adjust to environmental conditions. Because of their ecological importance, wide distribution, high availability throughout the year, sensitivity to environmental toxicants, and high capability of bioaccumulation, crustaceans are suitable as test-organisms in biomonitoring studies (Schilderman et al., 1999).

The results obtained in our study show the existence of tissue-specific changes of GSH content. Contents of tGSH were higher in the gills then in muscle and the hepatopancreas. These data may indicate a faster rate of GSH utilization or degradation in the former tissue, which could be responsible for the observed lower GSH content. Moreover, increase of GSH content in the gills compared with the hepatopancreas may be related to prevention of oxidative challenge in gill tissue (Dandapat et al., 2000). Earlier findings also suggest that the presence of high GSH content in red cells (Filho, 1996) and in gills (Marcon and Filho, 1999) of fishes is associated with attenuation of oxidative stress. The highest GSSG content was detected in muscle, the lowest in the hepatopancreas.

Aquatic organisms maintain high content of GSH in tissues (Thomas et al., 1982), and increased content has the function of protection (Thomas and Juedes, 1992). Also, high content of GSH could be a consequence of its increased synthesis due to high cysteine accessibility, which is necessary for GSH synthesis. The content of reduced GSH in muscle was approximate equal in the three species, while in the hepatopancreas and gills interspecies differences were detected. It should be emphasized that the pattern of alteration of GSH content in tissues is completely the same as in the case of tGSH, which is understandable because reduced GSH represents 9/10 of tGSH.

Published data indicate that GSH content

depends on the type of nutrition (Taylor et al., 1996). Also, in tissues of some organisms, the content of GSH is seasonally dependent. In the digestive gland of the mussel *Mytilus galloprovincialis*, the content of GSH is lowest in winter, when there is less food and the gonads are in a state of rest (Viarengo et al., 1991; Porte et al., 2000). GSH metabolism is regulated by several enzymes, so cellular GSH content is not constant and depends on the rates of synthesis, conjugation, and oxidation of GSH, as well as that of GR-dependent reduction of GSSG to GSH.

It has been found that many chemical substances can alter GSH content in tissues of different organisms. In liver of *Carassius auratus*, a decrease of GSH content was detected as a consequence of exposure to 2, 4-dichlorophenol for 40 days (Zhang et al., 2004). Other organic pollutants also provoke increase of GSH content, thereby providing extra protection against cytotoxic effects of biologically active molecules. Cellular GSH content increased after treatment with subtoxic Cd concentrations (Son et al., 2001a; Son et al., 2001b). This could provide the first line of defense against the influence of toxic heavy metals. Also, MacFarlane et al. (2006) found that heavy metals induce increase of GSH content in the crab *Parasesarma erythodactyla,* while naphthalene induces decrease of GSH content in the hepatopancreas, ovary, and gills of the crab *Scylla serrata* (Vijayavel and Balasubramanian, 2006). It has been established that GSH content and the GSH/GSSG ratio are related to the survival rate of mussels exposed to fenitrothion (Pena-Llopis et al., 2002). Although pollution influence on GSH content is regulated by a feedback mechanism and the liver, the GSSG/GSH ratio can be used as a potential biomarker in fish (Van der Oost et al., 1996).

The glutathione redox index represents a marker of the GSH/GSSG ratio, i.e., it defines the glutathione redox status of the cell. We found the highest GSH RI in the hepatopancreas and the lowest in muscle. In gills, the highest GSH RI was found in *O. limosus*, while in muscle it was recorded in *A. astacus*. Benzi et al. (1988) showed, that GSH content in the rat brain decreases and GSSG content increases with age, which means that the total GSH RI profile is age-linked and decreases with time.

In conclusion, the present study represents the first report treating the contents of tGSH, GSH, and GSSG and values of GSH RI in the investigated tissues (hepatopancreas, gills, and muscle) of two native (*A. astacus* and *A. torrentium*) and one introduced (*O. limosus*) freshwater crayfish species collected from three different Serbian rivers (the Southern Morava, the Krajkovačka, and Danube Rivers). The obtained results show tissue differences in the investigated species and indicate that the studied parameters can be considered a suitable biomarkers of the cellular glutathione redox status in freshwater crayfish species from different localities.

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ГЛУТАТИОН КАО ПОГОДАН БИОМАРКЕР У ХЕПАТОПАНКРЕАСУ, ШКРГАМА И МИШИЋИМА ТРИ ВРСТЕ СЛАТКОВОДНИХ РАКОВА

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Одређиван је садржај укупног глутатиона (tGSH), редукованог глутатиона (GSH), оксидованог глутатиона (GSSG) и глутатион редокс индекса (GSH RI) у хепатопанкреасу, шкргама и мишићима три врсте слатководних ракова: речног рака (*Astacus astacus*) из реке Јужна Морава, рака камењара (*Austropotamobius torrentium*) из Крајковачке реке и америчког рака (*Orconectes limosus*) из реке Дунав. Добијени подаци показују значајну ткивну и специјес специфичност испитиваних параметара: tGSH, GSH, GSSG и GSH RI у хепатопанкреасу, шкргама и мишићима код ракова. Наш рад представља прву студију ове врсте и показује да испитивани параметри могу бити погодни биомаркери ћелијског редокс статуса глутатиона код слатководних врста ракова.