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Content of cysteine, reduced and oxidized glutathione in spermatozoa of representatives of *Acipenseriformes* (*Acipenser baerii* and *A. ruthenus*) as well as teleosts (*Perca fluviatilis* and *Sander lucioperca*)

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Summary

Glutathione belongs to a vital intra- and extra-cellular protective antioxidant and is found almost exclusively in its reduced form. The ratio between its reduced and oxidized within cells is often used as a marker of cellular toxicity. The objectives of the study were to (i) determine both the reduced (GSH) and oxidized glutathione (GSSG) and cysteine (Cys) in the sperm of the *Acipenser baerii* and *Acipenser ruthenus*, as well as in *Perca fluviatilis* and *Sander lucioperca*, and (ii) to demonstrate the differences in concentration levels between representatives of acipenseriform and teleost species. High performance liquid chromatography with electrochemical detection was employed. The average content of the thiols determined in the sperm samples were as follows: *Acipenser baerii* (cysteine 55 ± 8 μg ml^{-1}; GSH 126 ± 19 μg ml^{-1}; GSSG 49 ± 7 μg ml^{-1}), *Acipenser ruthenus* (cysteine 62 ± 9 μg ml^{-1}; GSH 768 ± 115 μg ml^{-1}; GSSG 180 ± 16 μg ml^{-1}), *Sander lucioperca* (cysteine 251 ± 38 μg ml^{-1}; GSH 185 ± 28 μg ml^{-1}; GSSG 93 ± 14 μg ml^{-1}), *Perca fluviatilis* (cysteine 281 ± 42 μg ml^{-1}; GSH 496 ± 74 μg ml^{-1}; GSSG 138 ± 21 μg ml^{-1}). Based on the results obtained it can be concluded that this method is sensitive and selective for the determination of these compounds in real samples. Results revealed differences in cysteine content between species of the two systematic categories but also showed that ratios between GSH and GSSG can vary between species while potentially predict oxidative stress in fish sperm.

Introduction

Glutathione as a ubiquitous tripeptide consists of glutamic acid, while cysteine and glycine belong to the vital intra- and extra-cellular protective antioxidant. The reduced form is called 'reduced glutathione' (GSH) and is involved in detoxifying processes of xenobiotics and scavenging of 'reactive oxygen species' (ROS). In the process of scavenging of ROS two molecules of GSH are oxidised to form one molecule called oxidized glutathione (GSSG). Nevertheless, glutathione is found almost exclusively in its reduced form. This is mainly because the enzyme called glutathione reductase, which reverts it from its oxidized form, is constitutively active and inducible upon oxidative stress. In fact, the ratio of reduced to oxidized glutathione within cells is often used as a marker for assessing cellular toxicity (Meister and Anderson, 1983; Carelli et al., 1997; Townsend et al., 2003).

The oxidation stress induced by these xenobiotics can result in damages to DNA, RNA, membranes and other cell compartments. Spermatozoa have a high content of polyunsaturated fatty acids in their membranes and also contain high amounts of DNA, which are both sensitive to oxidative stress (Peris et al., 2007). The oxidative damage to the membranes and DNA in the sperm can influence the fertilization process and subsequently embryogenesis. The aim of our study was to test the suitability of a relatively simple method for determination of GSH, GSSG and the cysteine content in fish spermatozoa from two different systematic categories by using high performance liquid chromatography with electrochemical detection (HPLC-ED). To our knowledge thiols contents have not yet been determined in fish sperm and this is the reason why we focused particularly on the quantification of the ubiquitous thiols.

Material and methods

Chemicals and instruments

Cysteine (Cys), reduced (GSH) and oxidized (GSSG) glutathione, trifluoroacetic acid (TFA) and other chemicals used were purchased from Sigma-Aldrich (St Louis, MO, USA) unless noted otherwise. Stock standard solutions of the thiols (500 μg ml^{-1}) were prepared with ACS water (Sigma-Aldrich) and stored in the dark at −20°C. Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 μm Nylon filter discs (Millipore, Billerica, MA, USA) prior to HPLC analysis.

Fish sperm samples

Fish spermatozoa were sampled from *Acipenser baerii* (n = 4), *Acipenser ruthenus* (n = 9), *Perca fluviatilis* (n = 2) and *Sander lucioperca* (n = 3) bred at the Research Institute of Fish Culture and Hydrobiology, University of South Bohemia. The spermatozoa of *Acipenser* spp. were sampled in April, 2007. The spermatozoa were collected after hormonal stimulation of the mature fish by injecting suspensions of carp

pituitary. The collected sperm was placed on an ice bath for 2 h. Thereafter the samples were deep frozen at −85°C (Psenicka et al., 2007). The spermatozoa of *Perca fluviatilisi* and *Sander lucioperca* were sampled in March 2007 and were collected without hormonal stimulation. The collected sperm were also placed on an ice bath for 5 h. Thereafter the samples were also deep frozen at −85°C. Deep freezing did not affect sperm motility (DeGraaf and Berlinsky, 2004a,b). Homogenization of fish spermatozoa was carried out in an Ultra-Turax T8 homogenizer (IKA, Germany) for 1 min at 1500 rpm at 4°C. Furthermore, the sperm samples were kept at 2°C prior to analysis (time of storage was no more than 6 h).

Preparation of biological samples for analysis

The homogenized fish sperm samples were prepared by heat treatment. Briefly, the sample was kept at 99°C in a thermomixer (Eppendorf 5430, USA) for 15 min with occasional stirring, and thereafter cooled to 4°C. The denatured homogenates were centrifuged at 4°C, 15 000 ***g*** for 30 min (Eppendorf 5402). Heat treatment effectively denaturates and removes high molecular weight proteins from the samples (Kizek et al., 2001; Erk et al., 2002; Petrlova et al., 2006). The supernatants were stored at −20°C prior to analysis.

High performance liquid chromatography with electrochemical detection

The quantification of the thiols content was carried out according Potesil et al. (2005). High performance liquid chromatography was coupled with an electrochemical detector (HPLC-ED) system which consisted of two solvent delivery pumps operating in the range of 0.001–9.999 ml min^{-1} (Model 582 ESA Inc., Chelmsford, MA, USA), Metachem Polaris C18A reverse-phase column (150.0 × 4.6 mm, 3 μm particle size; Varian Inc., CA, USA) and a Coul-Array electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector included three flow cells (Model 6210, ESA, USA). Each cell consisted of four analytical cells. One analytic cell contained working carbon porous electrode, two auxiliary and two reference electrodes. Both, the detector and the column were temperature-controlled at 30°C. The sample (50 μl) was injected using an auto-sampler (Model 540 Microtiter HPLC, ESA, USA) at 4°C. Experimental HPLC-ED conditions were as follows – mobile phase composition: 80 mmol l^{-1} trifluoroacetic acid and methanol (3 : 1); flow of the mobile phase 0.8 ml min^{-1}, working electrodes potential at 900 mV (number of measurements = 3).

Under the experimental conditions the signals of cysteine were measured at retention times of approximately 4 min, GSH at 5 min and GSSG at 12 min. In the case of real sample measurements the shift of the retention time was about ± 2% (Fig. 1). GSH, GSSG and cysteine content were quantified as a sum of current responses from all working electrodes (Potesil et al., 2005; Petrlova et al., 2006).

pH determinations

The pH values were determined using a WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled via a personal computer program (MultiLab Pilot; Weilheim, Germany). The pH-electrode (SenTix-H, pH 0–14 / 3 M KCl) was regularly calibrated by sets of WTW buffers (Weilheim, Germany).

Results

Content of GSH, GSSG and cysteine in fish sperm samples

The concentrations of target molecules were proportional to the height of current responses. The results obtained showed the lowest content of cysteine in sperm of *A. baerii* (55 ± 8 μg ml^{-1}) followed by *A. ruthenus* (62 ± 9 μg ml^{-1}) (Fig. 2). The markedly highest levels of cysteine were determined in the samples of *S. lucioperca* (251 ± 38 μg ml^{-1}) and *P. fluviatilis* (281 ± 42 μg ml^{-1}) (Fig. 2 lower panel). Concentrations of GSH were highly variable between species and systematic categories. The lowest level of GSH was quantified in the sperm of *A. baerii* (126 ± 19 μg ml^{-1}) and *S. lucioperca* (185 ± 28 μg ml^{-1}). Higher content were measured in the samples obtained from *P. fluviatilis* (496 ± 74 μg ml^{-1}) and *A. ruthenus* (768 ± 115 μg ml^{-1}). Moreover the lowest concentrations of GSSG were determined in the samples of *A. baerii* (49 ± 7 μg ml^{-1}) and *S. lucioperca* (93 ± 14 μg ml^{-1}). Higher content were observed in the sperm of *P. fluviatilis* (138 ± 21 μg ml^{-1}) and *A. ruthenus* (180 ± 16 μg ml^{-1}) (Fig. 2). Ratios between GSH and GSSG content show that the level of GSH is about three times higher than level of GSSG, except *A. ruthenus*, in which the ratio is quite high (7.10) (inset in Fig. 2, insert panel).

Discussion

In this study, we demonstrated the general suitability of the method employed while also identifying for the first time the thiols content in fish spermatozoa of different systematic groups using high performance liquid chromatography with electrochemical detection. Particularly, we determined the content of one of the most important antioxidants (glutathione) in both, the reduced and oxidized form. Sasaki et al. (1996) showed that the low molecular mass and water soluble fractions from chum salmon male tissues exhibited considerable antioxidant potential, which the authors related to the GSH content and to other unknown peptides (Sasaki et al.,

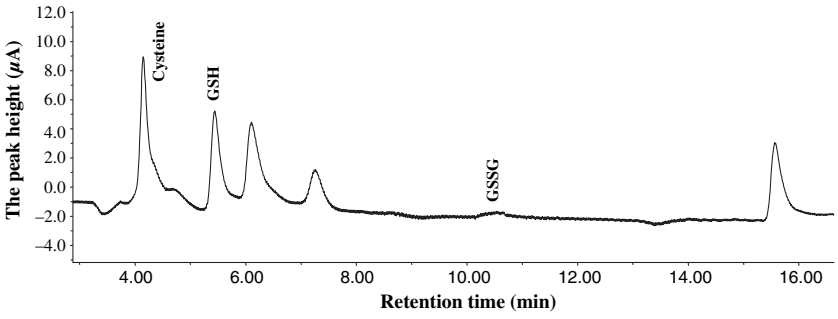

Fig. 1. Determining the thiols content in sperm samples of *Perca fluviatilis*. Example of a HPLC-ED chromatogram indicating the retention peaks for the identified substances Cysteine, GSH and GSSG

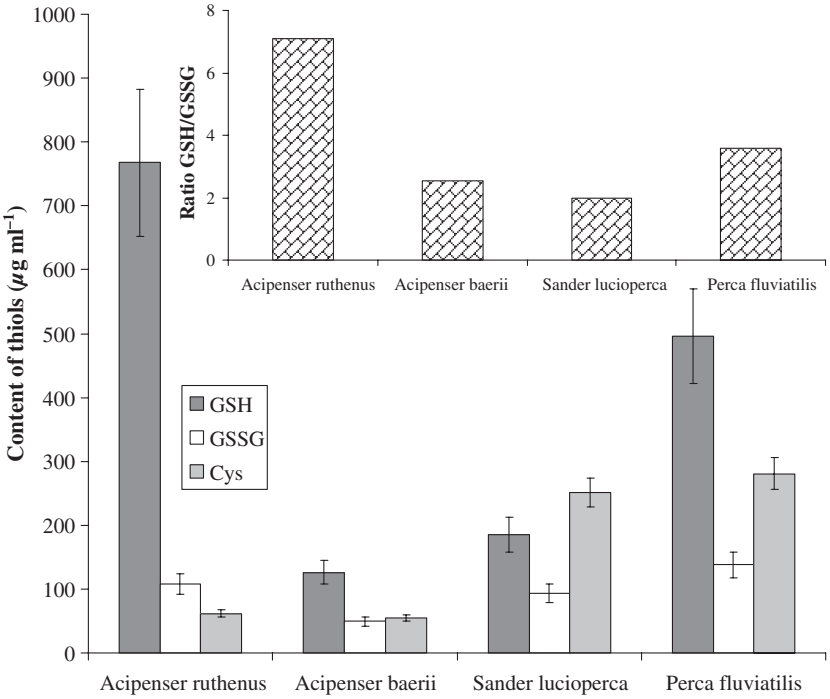

Fig. 2. Determined levels of cysteine and thiols (GSH and CSSG), in representatives of acipenseriform and teleost species (main panel). The resulting ratios for GSH / GSSG for the same four species are presented in the insert panel. Columns represent mean values and bars standard deviation (*A. ruthenus* n = 9; *A. baerii* n = 4; *P. fluviatilis* n = 2; *S. lucioperca* n = 3)

1996). We found that GSH content was relatively high in sperm of various fish species and assume that the content can be related to protection of sperm against abiotic stresses such as heavy metal induced stressors. This presumption is in good agreement with the results published by Ringwood and Conners (2000), which reported the effects of glutathione depletion on reproductive success in oysters (*Crassostrea virginica*). These authors found that GSH depletion did increase the susceptibility of developing embryos to metal toxicity (Ringwood and Conners, 2000; Ringwood et al., 2004). Moreover, Venturino et al. (2001) suggested exposure to thiols-depleting agents may induce alteration of organophosphate degradation in amphibian larvae. We believe that the method has a high potential to be used in stress research and its applicability should be explored further.

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