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Peroxidation and unsaturation indices as potential biomarkers of multifarious zinc and copper micro-supplementation in *Helix pomatia* L.☆

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ABSTRACT

The work is a continuation of two previous studies in which biomarker fatty acids (12 of 56 FA pools) were analysed in *Helix pomatia* L. after heterogeneous micro-supplementation of Zn and Cu (administered in five micro-doses in the form of salts and EDTA and lysine chelates). This time, peroxidation (PI) and unsaturation coefficients (UI) as biomarker were analysed. These indices were calculated based on the FA profile in the foot and hepatopancreas of snails. The correlation of frequently used oxidation status indicators of organisms (catalase - CAT, glutathione peroxidase - GPx, selenium-dependent peroxidase - se-GPx, superoxide dismutase - SOD, glutathione transferase - GST, glutathione reductase - GR, glutathione - GSH, carbonyl protein - CP, thiobarbituric acid reactive substances - TBARS) with the rarely used UI and PI ratios was analysed. It was found that the 12-week micro-exposure to Zn and Cu did not inhibit but rather stimulated antioxidative defence at a sufficient level to increase the values of peroxidation/unsaturation indices in comparison to the control groups. Induction of an opposite process to oxidation of fatty acids was demonstrated. Maximum activities and amounts of antioxidants as well as minima of protein and lipid decomposition were recorded in groups supplemented with 0.75 mg/l Zn and 1.0 mg/l Cu. The possibility of a direct use of fatty acids as well as peroxidation/unsaturation indices as sensitive and reproducible biomarkers of exposure and oxidative physiological status in snails was confirmed.

1. Introduction

Antioxidants produced by snails are involved in the high tolerance of these animals to environmental contamination with a variety of pollutants, including molluscicides (Kowalczyk-Pecka et al., 2017a) and metals (Chandran et al., 2005; Nowakowska et al., 2012) in both aquatic (Atli and Grosell, 2016) and terrestrial (El-Shenawy et al., 2012; Abdel-Halim et al., 2013) environments and to physiological stressors, waking from estivation (Nowakowska et al., 2014, 2016).

To date, a simple assumption has been adopted, according to which environmental and physiological stressors induce lipid peroxidation processes. The effects of these processes are determined with the use of standard physiological status biomarkers. These include enzymatic antioxidants (catalase - CAT, glutathione peroxidase - GPx, selenium dependent peroxidase - se-GPx, superoxide dismutase - SOD, glutathione transferase - GST, glutathione reductase - GR), non-enzymatic antioxidants (glutathione – GSH), and products of protein (CP) and lipid (TBARS) decomposition (Hermes-Lima and Storey, 1995; Hermes-Lima et al., 1998; Atli and Canli, 2008, 2010; Radwan et al., 2010a, 2010b). Assessment of the degree of oxidative stress based on the analysis of fatty acid profiles is a recently promoted idea (Kowalczyk-Pecka et al., 2017a). This study is in line with the trend of searching for alternative biomarkers of micro-exposure of organisms to environmental factors at the physiological response level.

In previous reports (Kowalczyk-Pecka et al., 2017b, 2017c) we demonstrated a rapid and reproducible response to heterogeneous micro-supplementation with copper, zinc, and molluscicides reflected in a panel of biomarker fatty acids in two snail tissues. Surprisingly, we detected an increase in the quantity of PUFA (Polyunsaturated Fatty Acid) and a decline in the level of SFA (Saturated Fatty Acid) and MUFA (Monounsaturated Fatty Acid) after administration of micro-doses of Cu and Zn compounds. We regarded this phenomenon as beneficial to

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snails, e.g. increased levels of bactericidal acids, and to potential consumers. The phenomenon occurs below certain threshold micro-doses of the supplement, above which the PUFA level begins to decline.

The research hypothesis assumed that even micro-doses of metals used as stimulators induce physiological response in snails, i.e. changes in lipid metabolism. Therefore, two research objectives were adopted. The first aim of the investigations was to confirm the effectiveness of FA as sensitive exposure biomarkers based on the analysis of the correlation of PI and UI with nine oxidation parameters. The second objective was to determine the basis of the interesting phenomenon of the increase in the PUFA content after micro-supplementation with zinc and copper and to provide an answer to a question whether this was a result of a "delay" of the peroxidation process or stimulation of antioxidative processes.

2. Material and methods

The protocol of metal supplementation and determination of fatty acids in *Helix pomatia* L. tissues was described in detail in two previous papers by Kowalczyk-Pecka et al. (2017b), (2017c).

Briefly, the experimental snail groups kept in strictly controlled laboratory conditions received five supplement doses of Zn and Cu salts ($ZnCl_2$ and $CuSO_4$, POCH S.A., respectively) or lysine and EDTA chelates at doses of 0.1, 0.25, 0.5, 0.75, or 1 mg of a solution of the supplemented metal /l redistilled H_2O. The groups were denoted as Zn/or Cu 0.1, Zn/or Cu 0.25, Zn/or Cu 0.5, Zn/or Cu 0.75, and Zn/or Cu 1.0. Another five groups receiving EDTA chelates were denoted as Zn + EDTA/or Cu + EDTA 0.1, Zn + EDTA/or Cu + EDTA 0.25, Zn + EDTA/or Cu + EDTA 0.5, Zn + EDTA/or Cu + EDTA 0.75, and Zn + EDTA/or Cu + EDTA 1.0. The lysine chelate-supplemented groups were designated as Zn + Lys/or Cu + Lys 0.1, Zn + Lys/or Cu + Lys 0.25, Zn + Lys/or Cu + Lys 0.5, Zn + Lys 0.75/or Cu + Lys, and Zn + Lys/or Cu + Lys 1.0.

2.1. Determination of peroxidation and unsaturation indices

Fatty acid esters were obtained according to PN-EN ISO 12966-1:2015-01 and PN-EN ISO 12966–2:2011 norms and AOAC Official Method 969.33 (1969). Based on the fatty acid analysis performed with the use of a Varian 3800 gas chromatograph, peroxidation (PI) and unsaturation (UI) indices were calculated. A formula developed by Hulbert et al. (2007) was employed:

$$\text{Peroxidation index (PI)} = 0.025 \times (\%\ \text{monoenoics FA}) + 1 \times (\%\ \text{dienoics FA}) + 2 \times (\%\ \text{trienoics FA}) + 4 \times (\%\ \text{tetraenoics FA}) + 6 \times (\%\ \text{pentaenoics FA}) + 8 \times (\%\ \text{hexaenoics FA})$$

$$\text{Unsaturation index (UI)} = 1 \times (\%\ \text{monoenoics FA}) + 2 \times (\%\ \text{dienoics FA}) + 3 \times (\%\ \text{trienoics FA}) + 4 \times (\%\ \text{tetraenoics FA}) + 5 \times (\%\ \text{pentaenoics FA}) + 6 \times (\%\ \text{hexaenoics FA})$$

2.2. Chemical reagents and apparatus

The chemical reagents used for the biochemical analyses were produced by Sigma Aldrich, BDH Chemicals, and Polskie Odczynniki Chemiczne (POCH S.A., now Avantar). All reagents and chemicals were of the highest purity grade.

A CARY 50 Spectrophotometer Varian part of Agilent Tech. (USA), an Eppendorf Microcentrifuge 5415 R an Elpan water bath shaker type 357 (Poland), an Omni GLH homogeniser A.G.A. Analytical (Poland), a centrifuge MPW-350R, MPW Medical Instruments Warsaw (Poland), were used for the determination of the oxidative status in the snails.

Snail foot and hepatopancreas tissues were homogenised and prepared using the method of Radwan et al. (2010a). The supernatants were used for the measurement of CAT, GPx Se-GPx, SOD, GR, and GST activities. The homogenate was used as a source of the MDA, CP, and GSH content. The blind samples contained only the reaction buffer without addition of either the supernatant or the homogenate.

For comparative analysis, the physiological status parameters were determined in both (foot and hepatopancreas) snail tissues.

2.3. Enzyme activity assay

Catalase activity (CAT) was measured using the method of Lartillot et al. (1988) and Bessey et al. (1946) and described in detail by Atli and Grosell (2016). The reaction buffer contained 100 mM phosphate buffer with pH 7.4 and 50 mM H_2O_2. The absorbance decline was examined at 240 nm for 1′ It was presented as μmol H_2O_2 /mg prot./min.

Glutathione peroxidase (GPx) and glutathione reductase (GR) activities were measured using the method of Livingstone et al. (1992) - GPx (100 mM phosphate buffer with pH 7.0, 2 mM GSH, 0.06 mM NADPH, 2 GR units, and 3 mM cumene hydroperoxide. The NADPH reduction was analysed at 340 nm for 1′) and Carlberg and Mannervik (1975) - GR (100 mM phosphate buffer with pH 7.0, 10 mM NADPH, and 1.3 mM GSSG), described in detail by Atli and Grosell (2016). The activities were calculated as μmol/mg prot. /min.

The activity of selenium-dependent peroxidase (Se-GPx) was measured according to Hermes-Lima and Storey (1995) described by Ramos-Vasconcelos and Hermes-Lima (2003)·H_2O_2 was used as a substrate for estimation of the enzyme activity. A quantitative test based on NADPH oxidation measured at absorbance of 340 nm was used. The Se-GPx activity was presented as mU/mg protein.

Superoxide dismutase (SOD) activity was measured by the indirect method proposed by McCord and Fridovich (1969) and described by Atli and Grosell (2016). Reaction buffer and other parameters of the analyses: 75 mM potassium phosphate buffer with pH 7.4, 0.1 mM EDTA, 10 mM cytochrome c, 25 mM hypoxanthine, and 56.6 mU/ml xanthine oxidase. The analysis involves inhibition of cytochrome c reduction at 550 nm for 1′. The SOD activity was presented as Unit/mg protein.

Glutathione transferase (GST) activity was quantified according to Habig et al. (1974) described in detail by Nowakowska et al. (2009). The product of the GSH and 1-chloro-2,4-dinitrobenzene (CDNB) reaction was determined at 340 nm for 3′.859 μl of phosphate buffer with pH 6.5, 50 μl of 100 mM GSH, and 50 μ of 20 mM CDNB in ethanol. The GST activity was presented as mmol/g protein/min.

2.4. Glutathione concentration assay

Glutathione (GSH) concentration was assayed according to the method of Ellman (1959) described in Nowakowska et al. (2009). Initial reaction mixture: 500 μl of tissue homogenate, 500 μl of TCA, 500 μl of 10 mM EDTA. Mixture CF 7′ /12.000 g. Reduced glutathione was determined with the use of 200 μl of the supernatant, 2.3 ml of deionized H_2O, 100 μl of EDTA, 300 μl of TRIS, and 100 μl of DTNB, (10 °C/ 10 min). Absorbance was measured at 412 nm. The glutathione concentration was expressed in μmol/g tissue.

2.5. Protein content

The protein contents were determined with the method of Bradford (1976) using bovine serum albumin as a standard. The methods were described in detail previously (Atli and Canli, 2008, 2010).