

Effects of desiccation on metamorphic climax in *Bombina variegata*: Changes in level and pattern of oxidative stress parameters

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Biochemical analyses

The concentration of total proteins was determined by Lowry et al. (1951). For the construction of the standard protein curve a series of bovine serum albumin (BSA) dilutions was used (the final concentration ranges between 100-1000 µg/mL). The procedure for protein concentration determination is based on the biuretic reaction of cupric ions (Cu²⁺) with peptide bonds of the protein in the alkaline environment and the reaction of the phosphomolybdenum-phosphoflavoformic reagent (Folin-Ciocalteu reagent) with aromatic amino acids tyrosine and tryptophan, which are constitutive parts of measured proteins. After binding to the peptide bonds, Cu²⁺ ions are reduced in the cuprous ions (Cu⁺) and the Cu⁺-protein complex is formed. This complex further reacts with the added Folin-Ciocalteu reagent to form a blue colored complex. The color intensity is proportional to the protein content and is measured spectrophotometrically at 500 nm.

The activity of SOD was determined by the adrenaline method (Misra and Fridovich, 1972), which is based on the ability of SOD to reduce spontaneous autoxidation of adrenaline in the adrenochrome in the alkaline environment. Autoxidation of adrenaline depends on the presence of O₂⁻. SOD present in the sample removes O₂⁻ and thus inhibits the autoxidation reaction. Reduction in the rate of adrenaline autoxidation is determined spectrophotometrically at a wavelength of 480 nm. The change in the absorbance is due to the pink-colored adrenaline. Solutions: 3 × 10⁻⁴ M adrenaline in 0.1 M HCl, carbonate buffer (0.05 M Na₂CO₃ + 10⁻⁴ M EDTA) pH 10.2 adjusted with 10% HCl and 8 mM KCN. Experimental procedure: 3 ml of carbonate buffer was poured into the glass cuvette, together with an

appropriate volume of the pre-adjusted adrenaline and the amount of sample that induces the adrenaline autooxidation inhibition ranging from 16.66% to 66.66%. To calculate the activity of SOD, the value of the sample absorption changes in the blank test (buffer and adjusted adrenaline) was used. The SOD activity unit is defined as the amount of enzyme which leads to 50% inhibition of adrenaline autooxidation in the linear portion of the change in absorbance per minute. The activity of SOD in the samples was expressed in units per milligram of protein (U/mg protein).

The activity of CAT was determined by the method described by Claiborne (1984). The method is based on monitoring the decomposition rate of H_2O_2 to H_2O and O_2 under the action of CAT. Reduction of the absorbance due to the consumption of H_2O_2 was detected spectrophotometrically at a wavelength of 240 nm. Experimental procedure: The H_2O_2 solution in phosphate buffer is adjusted so that the blank sample absorption at a wavelength of 240 nm is between 0.525 and 0.550. In a quartz cuvette, 1.5 ml of the adjusted solution of H_2O_2 in phosphate buffer was poured, and then the amount of sample which leads to a mean change in the absorbance in the range of 0.03 to 0.06 was added. In the sample starts the process of H_2O_2 decomposition reaction due to the presence of CAT. The reduction of the absorbance was monitored spectrophotometrically at 240 nm every 30 seconds for 3 minutes at a temperature of 25 °C. To calculate the CAT activity, a molar extinction coefficient for H_2O_2 ($43.6 \text{ M}^{-1} \text{ cm}^{-1}$), at a wavelength of 240 nm, was used. CAT activity unit is determined as the number of millimoles of H_2O_2 reduced per minute ($\text{mmol H}_2\text{O}_2/\text{min}$). The activity of enzymes in the tested samples is expressed in units per milligram of protein (U/mg protein).

The GSH-Px activity was measured by the method developed by Tamura et al. (1982). The principle of the method is based on the coupled activity of GSH-Px (catalyses the oxidation of GSH in GSSG with the reduction of organic hydroperoxides) and GR (enables the reduction of GSSG in GSH with the oxidation of NADPH as a coenzyme). The organic peroxide tert-butyl hydroperoxide is added to the reaction mixture, with NADPH and GR. The activity of the GSH-Px enzyme was detected by spectrophotometric monitoring of NADPH oxidation into NADP^+ . Into quartz cuvette were poured: 1.6 mL of H_2O (or less volume depending on the amount of added sample), 0.3 mL of 1mM GSH, 0.6 mL of 0.2 mM NADPH, 0.1 mL of 1mM NaN_3 (which inactivated CAT), 0.1 mL of 1 mM EDTA, 0.3 mL 0.5M phosphate buffer (pH 7.0), 0.1 mL of 0.03 M tert-butyl hydroperoxide, adequate amount of sample and 5 μL GR. Decrease of the absorbance was monitored spectrophotometrically at 340 nm every 30 seconds for 3 minutes at a temperature of 25 °C. The activity of GSH-Px was determined according to the blank, using a molar extinction coefficient of $6.22 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ for NADPH at 340 nm. The GSH-Px enzyme activity unit was defined as the number of oxidized nanomoles of NADPH per minute ($\text{nmol NADPH}/\text{min}$), and the activity of GSH-Px in the tested tissues is expressed in units per milligram of protein (U/mg protein).

To determine the activity of GR in the tested samples the method according to Glatzle et al. (1974) was used. This method is established on the ability of GR to catalyze the GSSG reduction into GSH with the oxidation of coenzyme NADPH to NADP^+ . In the reaction mixture, the

GSSG and NADPH are added, and GR activity is measured spectrophotometrically by reducing the NADPH concentration.

Experimental procedure: In a quartz cuvette 0.6 mL 0.5 M phosphate buffer (pH 7.4), 0.1 mL 2 mM GSSG, 0.1 mL 0.5 mM EDTA, 2 mL H₂O (or a smaller volume depending on the volume of the sample) and 0.1 mL 0.1 mM NADPH were added, and at the end adequate amount of the sample. The absorbance was measured at a wavelength of 340 nm every 30 seconds for 3 minutes at a temperature of 25 °C. To calculate the GR activity, a molar extinction coefficient for NADPH at 340 nm of $6.22 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$ was used. The unit of activity of the enzyme GR is defined as the number of nanomoles oxidized NADPH per minute (nmol NADPH/min). The activity of this enzyme in the tested samples is presented in units per milligram of protein (U/mg protein).

The activity of the phase II biotransformation enzyme - GST was measured by the method described by Habig et al. (1974). The principle of the method is based on the ability of the GST to catalyze the reaction of binding 1-chloro-2,4-dinitrobenzene (CDNB) to the sulfhydryl group of cysteine that is a part of the tripeptide GSH, thereby forming a CDNB-GSH conjugate. The rate of absorption due to the formation of CDNB-GSH conjugate is directly comparative to the activity of the GST in the sample. Experimental procedure: 2 ml of H₂O poured into the quartz cuvette (or a smaller volume depending on the amount of sample) were followed by 0.1 mL 25 mM CDNB in 95% ethanol, 0.6 mL of 0.5 M phosphate buffer (pH 6.5), 0.3 mL of 20 mM GSH and adequate amount of sample. The absorbance was monitored spectrophotometrically at a wavelength of 340 nm every 30 seconds for 3 minutes at a temperature of 25 °C. The GST activity was determined according to the blank, using the molar extinction coefficient for the CDNB-GSH conjugate at 340 nm of $9.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$. The absorbance is detected spectrophotometrically at a wavelength of 340 nm. The unit of activity of the enzyme phase II biotransformation GST is expressed as the number of nanomoles of the CDNB-GSH conjugate formed per minute (nmol CDNB-GSH/min). GST activity is given in units per milligram of protein (U/mg protein).

The method described by Griffith (1980) was used to measure the concentration of total GSH in the test samples. This method is based on a cyclic enzymatic process: 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) oxidizes GSH, whereby GSSG and 2-nitro-5- thiobenzoic acid (TNB) are formed, and then the GR enzyme reduces GSSG in GSH with NADPH coenzyme oxidation. The formation rate of the yellow colored TNB compound is monitored and is proportional to the concentration of total GSH in the sample. Experimental procedure: 0.5 mL tissue sonicates and 0.25 mL of 10% sulfosalicylic acid (for protein precipitation in the sample) were poured in microcentrifuge tubes. After centrifugation for 10 minutes at 5000 rpm, the obtained supernatant was used to determine the concentration of GSH. 0.1 mL 6 mM DTNB, sample, 0.7 mL 0.3 mM NADPH, H₂O to 1 mL of the reaction mixture and 5 µL of GR were poured in a quartz cuvette. For the standards, instead of the sample, the appropriate volumes of standard solutions with the known concentration of GSH were added. An increase of the absorbance is monitored spectrophotometrically at a wavelength of 412 nm every 30 seconds for 3

minutes at a temperature of 25 °C. The absorbance is determined spectrophotometrically at 412 nm. The concentration of total GSH in the sample is expressed in nanomoles per gram of tissue (nmol of GSH/g tissue).

The concentration of free -SH groups in the tested samples was measured by the method of Ellman (1959). DTNB oxidizes free -SH groups present in the sample, whereby mixed disulfides and yellow colored TNB are formed. Experimental procedure: In the cuvette 0.5 ml of sample was added, 0.5 mL of 0.1 M phosphate buffer (pH 7.3) and 0.2 mL of DTNB. After incubation for 10 minutes at room temperature, the absorbance at a wavelength of 412 nm was read. To calculate the concentration of free SH groups, a molar extinction coefficient of $14150 \text{ M}^{-1} \text{ cm}^{-1}$ was used. The concentration of free -SH groups in the sample is determined according to the blank and proportion of formed concentration of TNB. The absorbance is measured spectrophotometrically at 412 nm wavelength. The concentration of free -SH groups in the sample is expressed in micromoles of -SH group per gram of tissue ($\mu\text{mol SH/g tissue}$).

Thiobarbituric acid reactive substances (TBARS) concentrations as a marker of lipid peroxidation process (LPO)- oxidative damage were estimated according to the method of Rehnrcrona et al. (1980). The content of TBARS formed spontaneously was measured upon treating the samples with cold thiobarbituric acid reagent (10% trichloroacetic acid, 0.6% thiobarbituric acid) and subsequent heating at 100°C.

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Table 1. P values of between experimental units (containers) comparison for each stage within treatments for oxidative stress parameters (One way ANOVA).

	42		44		46	
	desiccation	control	desiccation	control	desiccation	control
SOD	0.392	0.827	0.856	0.568	0.822	0.694
CAT	0.299	0.961	0.087	0.761	0.647	0.454
GSH-Px	0.38	0.563	0.718	0.694	0.96	0.412
GR	0.059	0.279	0.354	0.569	0.345	0.522
GST	0.428	0.595	0.221	0.36	0.083	0.066
GSH	0.863	0.577	0.22	0.921	0.316	0.774
SH	0.329	0.857	0.869	0.32	0.26	0.608
LPO	0.203	0.836	0.512	0.419	0.53	0.713

Table 2. Multiple pairwise comparisons on the oxidative stress parameters (CAT, GSH-Px, GST and SH groups) with significant treatment x stage interaction. We highlighted all interactions that were according to the aim of this study (blue- comparisons between stages in constant water-control treatment; yellow- between treatments for each stage; green- between stages in decreasing water level- desiccation treatment) while significant *p* values were bold.

Contrast	CAT	GSH-Px	GST	SH
42 x control vs 44 x control	0.0008	0.2059	0.1356	0.2417
42 x control vs 46 x control	0.1216	0.0005	0.3969	< 0.0001
44 x control vs 46 x control	0.0333	< 0.0001	0.0081	0.0006
42 x control vs 42 x desiccation	0.0712	0.0603	0.2812	0.7836
44 x control vs 44 x desiccation	< 0.0001	0.2775	0.1708	0.1464
46 x control vs 46 x desiccation	0.9276	0.0003	0.0369	0.0367
42 x desiccation vs 44 x desiccation	0.0002	0.0797	0.3405	0.0443
42 x desiccation vs 46 x desiccation	0.1841	0.1399	0.8881	0.1409
44 x desiccation vs 46 x desiccation	0.0074	0.8951	0.2865	0.6462
42 x control vs 44 x desiccation	0.0020	0.8153	0.8774	0.0228
42 x control vs 46 x desiccation	< 0.0001	0.7342	0.2363	0.0835
44 x control vs 42 x desiccation	0.1596	0.3468	0.7887	0.3895
44 x control vs 46 x desiccation	0.0023	0.4133	0.9217	0.3947
46 x control vs 42 x desiccation	0.6504	< 0.0001	0.0444	0.0002
46 x control vs 44 x desiccation	0.0544	0.0001	0.2968	0.0838