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Original Research

Expression of Superoxide Dismutase Isoforms in *Desmodesmus subspicatus* Cells Exposed to Anthropogenic Contaminants

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Abstract

Expression of superoxide dismutase (SOD) isoforms was assessed as a biochemical marker, allowing to estimate the toxicity of chemicals toward a population of microalga *Desmodesmus subspicatus* (Chlorophyta). The effect of low (EC_{10}) and elevated (EC_{50}) doses of cadmium chloride (Cd), anthracene (ANT), and chloridazon (CHd) on SOD activity and isoform profile, as well as the amount of biosynthesized protein of ascribed to chloroplast (Fe-SOD) and cytosol (Cu/Zn-SOD) superoxide dismutase, were analyzed. Activities of Mn-SOD, Cu/Zn-SOD, and two Fe-SOD isoforms were determined densitometrically by native polyacrylamide gel electrophoresis and specific staining for SOD activity. Fe-SOD and Cu/Zn-SOD protein amount was analyzed by the semi-quantitative blot technique. The toxicants tested had no effect on the activity of Fe-SOD 1 isoform, whereas Fe-SOD 2 activity increased significantly. Simultaneously, the amount of Fe-SOD 1 protein increased after low contaminant doses treatment, whereas the amount of Fe-SOD 2 increased in cells exposed to elevated Cd, ANT, and CHd concentrations. A significant increase in Cu/Zn-SOD protein amount was observed in cells treated with EC_{50} doses of ANT and Cd, as well as CHd in concentrations corresponding to the EC_{10} value. At the same time, the activity of Cu/Zn-SOD increased in *D. subspicatus* cells exposed to an elevated dose of CHd. We have found that in the cells of *D. subspicatus*, the induction of Fe-SOD 1 and Cu/Zn-SOD Csd2 biosynthesis responds to xenobiotic-mediated stress level. The increase in the amount of chloroplastic Fe-SOD 1 and Cu/Zn-SOD Csd2 isoforms may be a sensitive biochemical marker of stress conditions, revealing the effects of low doses of aquatic contaminants on green algae.

Keywords: SOD isoforms, anthracene, cadmium, chloridazon, *Desmodesmus subspicatus*

Introduction

Cadmium (heavy metal), anthracene (polycyclic aromatic hydrocarbon, PAH), and chloridazon (triazine herbicide) are contaminants of our particular interest due to their elevated concentrations reported in the Gdańsk Bay region [1]. Analysis of air and surface water quality indicates that, in the last decade, inputs of total PAH and herbicides increased by 10 and 16%, respectively, and overcame the permissible level by over 15% [1]. At the same time, total inputs of Cd decreased 24%, but over 0.0015 $kg \cdot ha^{-1}$ is still annually deposed of in soil and water bodies, which

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results in exceedance of Cd permissible levels in over 25% [1]. In aquatic ecosystems elevated concentrations of these contaminants may lead to alternative growth, photosynthesis, and other physiological processes of unicellular planktonic algae – the basal element of the trophic food chain.

In view of its high affinity to sulphydryl groups, cadmium is regarded as a metal blocking essential functional groups in biomolecules, which results in the inhibition of various metabolic processes in cells [2]. The toxicity of anthracene results from its photoconversion to an oxidized, often more hazardous form [3], and/or from photosensitization, as well as modification of properties of lipid-rich membranes [4]. Chloridazon is an organochloride herbicide blocking the electron transport from Q_b to PQ in PS II by binding to D1 protein and decreasing its turnover rate [5].

Herbicides, aromatic hydrocarbons, and heavy metals are classes of chemical contaminants generally known as enhancers of reactive oxygen species (ROS) production [6]. The imbalance between ROS production and scavenging often leads to a phenomenon called oxidative stress [6]. The term oxidative stress refers to the overproduction of ROS such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), singlet oxygen ($O_2(^1\Delta_1)$), and hydroxyl radical (OH*). Plants have evolved various antioxidative response systems against ROS: from low-molecular-weight antioxidants, such as ascorbate, reduced glutathione, or tocopherols, to ROS-scavenging enzymes like superoxide dismutases, catalases, or peroxidases [7].

Superoxide dismutases constitute a family of metaloenzymes that catalyze disproportionation of two superoxide anions ($O_2^{\cdot-}$) to H_2O_2 and O_2, and are described as the "first line of defence" against ROS. SODs are classified into three groups according to their metal cofactor: copper-zinc (Cu/Zn-SOD), iron (Fe-SOD), and manganese (Mn-SOD) [8]. There are two distinct groups of Fe-SODs located in plant chloroplasts: the first one is a homodimer formed from two identical 20 kDa subunits, and the second is a tetramer of equal 80-90 kDa subunits [7]. Mitochondrial Mn-SOD is either a homodimeric or homotetrameric enzyme with one Mn atom per subunit. Two main Cu/Zn-SOD groups are revised in plant cells: one is a cytosolic and periplasmatic form (homodimeric), and the other is chloroplastic and extracellular (homotetrameric) [9]. Unlike other organisms, plants have multiple SOD forms: one type of SOD may be present in more than one isoform that has the same catalytic specificity, but different kinetic properties and different migration rates on a gel [10], thus the term "isoform" used in a variety of ways, refers to the total number of SOD bands observed, with no genetic interpretation [11].

The aim of this work was to evaluate the role of SOD isoforms in the protection of algal cells against contaminants that induce oxidative stress with a focus on the differences in SOD expression under low and elevated doses of cadmium, anthracene, and chloridazon.

Materials and Methods

Cultures

Desmodesmus subspicatus (Chlorococcales, Chlorophyta) strain 86.81 was obtained from Sammlung von Algenkulturen, University of Göttingen, Germany (SAG).

Anthracene (high purity, Aldrich Chemicals Co., USA) was dissolved in dimethylsulfoxide (DMSO) (Acros Organics, Belgium). DMSO (0.1% v/v) had no significant effect on the growth of *Desmodesmus* cells [12]. Cadmium ($CdCl_2 \cdot H_2O$, analytical grade; Merck, Germany) was dissolved in redistilled water. Chloridazon (Redel de Hanen, Germany) was used as commercial grade with 88% content of the active substance. The herbicide was dissolved in redistilled water. Final concentrations of chemicals in cultures corresponded to previously estimated concentrations causing 10% (EC_{10}) and 50% (EC_{50}) growth rate reduction in relation to the control cultures and stand as follows: ANT: EC_{10} – 0.08 $mg \cdot dm^{-3}$, EC_{50} – 0.44 $mg \cdot dm^{-3}$; $CdCl_2$: EC_{10} – 16.43 $mg \cdot dm^{-3}$, EC_{50} – 20.16 $mg \cdot dm^{-3}$; and CHd: EC_{10} – 2.29 $mg \cdot dm^{-3}$, EC_{50} – 5.88 $mg \cdot dm^{-3}$.

The batch cultures were set up by dilution of the preculture to an initial density of 1×10^6 cells ml^{-1}. Algae were grown on mineral Bold Basal Medium (BBM) in 200 cm^3 glass test-tubes submersed in a thermostated water bath at a constant 30ºC, under continuous fluorescent light from TLD 58W/54 lamps (Philips) providing 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The cultures were aerated with a sterile gas mixture containing 2.5% CO_2 passed through a filter (Sartorius 2000; 0.2 μm PTFE). Chemicals were added to the cultures when set up. The control and chemically treated algae were sampled for analysis after 24 hours.

Enzyme Isolation and Assays

SOD isolation procedure, enzyme activity determination and identification of isoforms visualized in PAGE analysis were performed as described by Pokora et al., [13] and Tukaj and Pokora [14] using a method based on NBT reduction, according to Beauchamp and Fridovich [15].

The protein content in algal extracts was determined according to Bradford [16], with bovine serum albumin as a standard.

The amount of SOD protein in *Desmodesmus* cells was analyzed via semi-quantitative western blotting followed by the chemiluminescent method. Samples containing 20 μg of total protein were separated in a 12.5% poliacrylamide gel (SDS-PAGE) and transfered to the nitrocellulose membrane in a semi-dry system (2 h, 50 mA). The membrane was blocked overnight in 5% fat-free milk in PBS buffer. After the washing step, the membrane was incubated overnight in primary antibody solution at a dilution of 1:1000 (rabbit polyclonal IgG anti-Fe-SOD or anti-Cu/Zn-SOD, Agrisera, Sweden), and next incubated in secondary HRP-conjugated antibodies (goat anti-IgG rabbit coupled with HRP, #A9169-2ML, Sigma) at a dilution of 1:2000 for 2 h at room temperature. Detection was performed using a

Table 1. Total SOD activity (U·mg^{-1} protein) determined spectrophotometrically (A) and densitometrically (B) in cells of *D. subspicatus* treated for 24 h with the EC_{10} and EC_{50} values corresponding to concentrations of anthracene, cadmium chloride, and chloridazon.

	Control	ANT EC_{10}	ANT EC_{50}	Cd EC_{10}	Cd EC_{50}	CHd EC_{10}	CHd EC_{50}
A	2.75±0.32	3.03±0.36	2.74±0.32	3.31±0.39	2.40±0.28	2.33±0.28	3.73±0.44
B	2.24±0.23	2.37±0.26	2.38± 0.31	2.44±0.25	2.58±0.28	2.47±0.24	3.03*±0.32

Values marked with asterisks are different from the control at p>0.05.

chemilumunescent substrate for horseradish peroxidase (ECL+ PLUS West Pico; Amersham Pharm. Biotech.) with autofluorography on high sensitivity photo film. The films were scanned after development and fixing. Densitometric analysis was performed using Quantity1D (Bio-Rad) software. The activity of a separate SOD isoform band was calculated by comparison of the relative band intensity mm^{-2} to the one obtained for Cu/Zn-SOD reference pattern of defined activity of 4.408 U·mg^{-1} protein (Sigma-Aldrich). Molecular weights were calculated using Quantity 1D software (Bio-Rad) according to MW marker Fermentas SM0441.

Statistical Analysis

Statistical analysis was performed using an MS Excel 2000 program (Microsoft). Standard deviation (SD), standard error (SE) and Student's t-test were evaluated at a significance level of $p<0.05$.

Results

SOD Activity

The total SOD activity determined spectrophotometrically was 2.75 U·mg^{-1} protein in the control and 2.33 to 3.73 in contaminant-treated cells, which were not significantly different statistically from the control (Table 1). Densitometric analysis of PAGE separated SOD bands intensity allowed us to estimate the activity of each previously indentified SOD isoform [13], i.e. one Mn-, one Cu/Zn-, and two Fe-SOD (Fig. 1). The activity of total cellular SOD in the control cells was 2.24 U·mg^{-1} protein (Table 1). Individual activities of each SOD isoform reached 0.5 to 1.1 U·mg^{-1} protein, and their participation in total enzyme activity was equal (Table 2). Although no statistically significant effects of toxicants applied on total SOD activity occurred (Table 1), the effects on enzyme isoform activities were obvious. EC_{50} doses of ANT and CHd declined, while

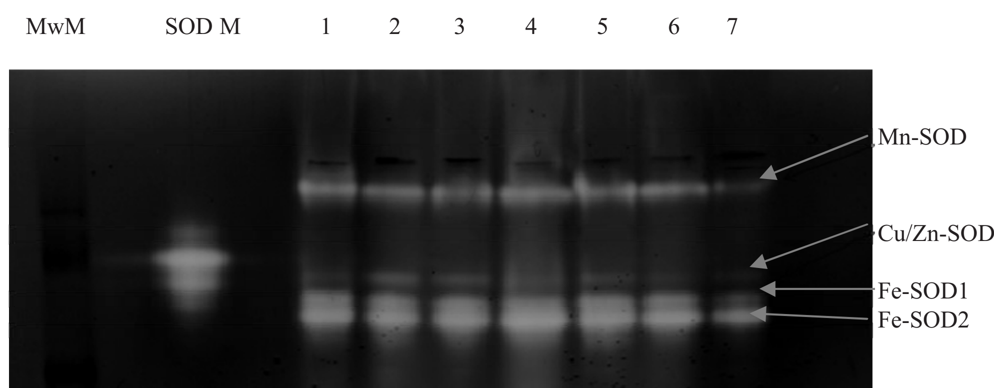

Fig. 1. SOD isoenzymes profile obtained for the control and treated for 24 h with anthracene, cadmium chloride, and chloridazon cells of *D. subspicatus*. PAGE analysis in a 12.5% gel stained for SOD activity. 50 µg protein per line; MwM – molecular weight marker, SOD M – Cu/Zn-SOD reference pattern of defined activity 4.4 U·mg^{-1} protein (Sigma-Aldrich); Lines: 1: control, 2: ANT EC_{10}, 3: ANT EC_{50}, 4: Cd EC_{10}, 5: Cd EC_{50}, 6: CHd EC_{10}, 7: CHd EC_{50}.

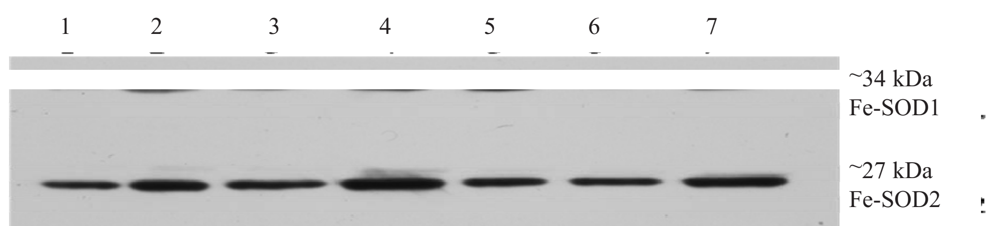

Fig. 2. Western blot detection of Fe-SOD1 and Fe-SOD2 isoforms in the control and treated for 24 h with anthracene, cadmium chloride, and chloridazon cells of *D. subspicatus*. 20 µg protein per line; Lines: 1: control, 2: ANT EC_{10}, 3: ANT EC_{50}, 4: Cd EC_{10}, 5: Cd EC_{50}, 6: CHd EC_{10}, 7: CHd EC_{50}.

Table 2. Densitometrically determined activity of SOD isoforms in cells of *D. subspicatus* treated for 24 h with EC_{10} and EC_{50} values corresponding to the concentrations of anthracene, cadmium chloride, and chloridazon. SOD activity was estimated densitometrically after PAGE analysis and SOD activity staining.

	Mn-SOD	Cu/Zn-SOD	Fe-SOD 1	Fe-SOD 2
Control	0.71±0.02	0.49±0.08	0.58±0.17	0.46±0.09
ANT EC_{10}	0.67±0.12	0.63*±0.13	0.59±0.09	0.48±0.10
ANT EC_{50}	0.56 *± 0.15	0.70*±0.14	0.57±0.09	0.54*±0.09
Cd EC_{10}	0.71±0.09	0.57±0.11	0.61±0.14	0.55*±0.12
Cd EC_{50}	0.89±0.14	0.70*±0.21	0.48*±0.08	0.51±0.08
CHd EC_{10}	0.75±0.12	0.53±0.14	0.61±0.11	0.57*±0.08
CHd EC_{50}	0.56*±0.08	1.35*±0.32	0.55±0.09	0.57*±0.06

Values marked with asterisks are different from the control at p>0.05.

Table 3. Results of a semi-quantitative analysis of Fe-SOD 1 and Fe-SOD 2 protein content in cells of *D. subspicatus* treated for 24 h with EC_{10} and EC_{50} values corresponding to the concentrations of anthracene, cadmium chloride, and chloridazon. Values are given as the multiplicity of the relative protein amount detected in control cells.

	Control	ANT		Cd		CHd	
		EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}
Fe-SOD 1	1.00	4.4*±0.1	3.2*±0.1	10.6*±0.2	7.9*±0.2	4.4*±0.1	12.3*±0.1
Fe-SOD 2	1.00	1.6*±0.1	1.5*±0.1	2.4*±0.1	1.0±0.1	1.1±0.1	1.9*±0.1

Values marked with asterisks are different from the control at p>0.05.

Cd stimulated the activity of Mn-SOD. Toxicants at EC_{10} concentrations had no effect on this isoform activity. In all treated cells the activity of Cu/Zn-SOD increased and the stimulation effect was the most significant (270% of control) in cells exposed to CHd at EC_{50} concentration. Toxicants revealed different effects on two identified Fe-SOD isoforms: there was no effect on Fe-SOD 1, while the activity of Fe-SOD 2 was stimulated in all treatment variants.

SOD Protein Content

Western-blot analysis allowed to identify two Fe-SOD isoforms, revealing the specific reaction with the *C. reinhardtii* originated antibodies recognizing the N-terminal polypeptide chain fragment (Agrisera, Sweden) (Fig. 2). The amount of protein (band area and intensity) of Fe-SOD 2 was nearly ten times higher than that of Fe-SOD1 (Fig. 2). In the contaminant-treated cells, a significant increase of both Fe-SOD isoform protein amount in relation to the control cells was detected (Table 3). The increase in Fe-SOD 1 isoform protein amount was most evident in cells treated with Cd (8-10-fold) and CHd at EC_{50} concentration (12 fold). On the other hand, the effect of toxicant treatment on the Fe-SOD 2 isoform was less significant, when 1.5-2-fold greater amounts of this protein were found in cells treated with ANT and Cd at EC_{10} and CHd at EC_{50} concentration. Two Cu/Zn-SOD: 19 and 16 kDa proteins were identified (Fig. 3). The increase in Csd1 isoform amount was detected in all chemically treated cells, but the effect was most pronounced in cells treated with ANT and Cd at EC_{50} and CHd at EC_{10} concentration (Table 4). In the same treatment variants, the presence of Csd2 isoform was confirmed and Cd at EC_{50} concentration caused a 7-fold increase of Csd2 protein content in *D. subspicatus* cells.

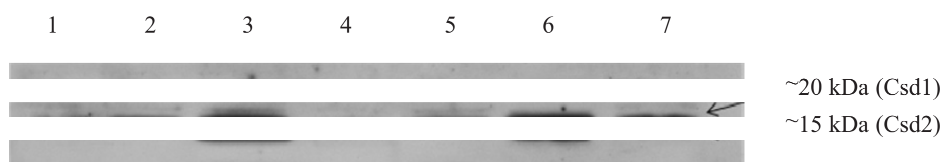

Fig. 3. Western blot detection of Cu/Zn-SOD1 (Csd1) and Cu/Zn-SOD2 (Csd2) isoforms in the control and treated for 24 h with anthracene, cadmium chloride, and chloridazon cells of *D. subspicatus*. 20 µg protein per line; Lines: 1: control, 2: ANT EC_{10}, 3: ANT EC_{50}, 4: Cd EC_{10}, 5: Cd EC_{50}, 6: CHd EC_{10}, 7: CHd EC_{50}.

Table 4. Results of a semi-quantitative analysis of Cu/Zn-SOD Csd 1 and Csd 2 protein content in cells of *Desmodesmus subspicatus* treated for 24 h with EC_{10} and EC_{50} values corresponding to the concentrations of anthracene, cadmium chloride, and chloridazone. Values are given as the multiplicity of the relative protein amount detected in control cells.

	Control	ANT		Cd		CHd	
		EC_{10}	EC_{50}	EC_{10}	EC_{50}	EC_{10}	EC_{50}
SOD Csd 1	1.00	1.42*±0.09	4.20*±0.07	3.64 *±0.14	6.94*±0.06	6.12*±0.17	2.15 *±0.17
SOD Csd 2	1.00	n.d.	6.66*±0.25	n.d	0.69*±0.18	2.22*±0.12	n.d.

Values marked with asterisks are different from the control at P>0.05. n.d.: not detected.

Discussion

The conformity of SOD activities obtained in the spectrophotometrical measurements and densitometric analysis of electrophoretically separated protein validate the usefulness of a densitometric method rather seldom used for SOD activity determination [17]. Herein, no significant contaminant-treatment effects on total SOD activity were detected when the stimulation or inhibition of SOD isoforms activities was observed. Most probably it would have been lost, if only spectrophotochemical total SOD activity determination had been applied. The fact that ANT, Cd, and CHd (known as oxidative stress enhancers) [12, 14] had no effect on total SOD activity was somehow surprising. The effects on particular enzyme isoforms indicated differences among cell compartments in cellular oxidative-stress-related response toward low and elevated doses of ANT, Cd, and CHd. For Fe-SOD and Cu/Zn- SOD, enzymatic activity assessment was followed by western blotting analysis, which, to our knowledge, has not previously been done with *Desmodesmus* genus. In western blotting analysis two Fe-SOD isoforms, revealing a specific reaction with the *Chlamydomonas reinhardtii* originated antibodies recognizing N-therminal polypeptide chain fragment were indentified, that were described as analogous to Fe-SOD 1 and Fe-SOD 2 *Arabidopsis thaliana* proteins [18]. According to the MW analysis two Cu/Zn-SOD, 19 and 16 kDa proteins were identified (Fig. 3). Cu/Zn-SODs of the similar MW in *A. thailana* are described as cytosolic Csd1 and chloroplastic Csd2 isoforms [19].

All contaminants examined, applied at low and elevated concentrations, stimulated the activity of Cu/Zn-SOD isoform ascribed to cytosol. The activities of mitochondrial and one of chloroplastic Fe-SOD isoforms were affected only by EC_{50} doses of the chemicals. For ANT and CHd it might be linked to their unspecific lipid membrane interactions [4, 20] and for Cd might result from direct inhibition of the respiratory chain [2]. Semi-quantitative western blotting analysis indicated the presence of cytosolic and chloroplastic Cu/Zn-SOD isoforms which, due to their similar MW, was undetectable on PAGE gel. All xenobiotics applied to algal cultures resulted in an increase in cytosolic Cu/Zn-SOD protein content, whereas the biosynthesis of chloroplastic Csd2 isoform was stimulated mainly by EC_{50} concentrations of the chemicals. The increase in the Csd2 level in cells treated with EC_{10} doses by chemicals, potentially (ANT) [4] or directly (CHd) [20] interacting with PS II, indicate that this SOD isoform is biosynthesized in response to chloroplast-originated oxidative stress.

Both PAGE and western blotting analysis indicated the presence of two chloroplastic Fe-SOD isoforms in *D. subspicatus* cells. Although there are no significant differences in the Fe-SOD isoform activity detected on PAGE gel, the western blotting analysis indicated nearly a ten-fold higher amount of Fe-SOD 2, with a simultaneous slight effect of contaminants on the protein level. The biosynthesis of second Fe-SOD 1 isoform is obviously induced under xenobiotic-mediated stress conditions (10-12-fold increase), even when low (EC_{10}) doses of chemicals were applied. The results obtained from the PAGE analysis and western blotting semi-quantitative measurements suggest that in the cells of *D. subspicatus* one of the Fe-SOD isoforms is expressed constitutively, when the enzyme activity reaches a relatively moderate level, sensitive to highly elevated doses of contaminates applied. Studies on expression regulation of chloroplastic Fe-SOD from *Lingulodinium polyedrum* (*Dinoflagellate*) showed that the light:dark rhythm of SOD activity is not related to changes in Fe-SOD mRNA levels, indicative of translational regulation. By contrast, conditions of metal-induced oxidative stress resulting in higher levels of Fe-SOD transcripts suggest that transcriptional control is responsible for increased protein and activity levels [21]. Authors indicated a daily (circadian) and metal-induced up-regulation of Fe-SOD expression in *L. polyedrum* mediated by different regulatory pathways, which somehow confirms the existence of at least two different regulatory mechanisms of Fe-SOD expression in algae. We had also observed a significant divergence between Fe-SOD protein amounts and enzyme activity due to the fact that hydrogen peroxide generated by SOD, when scavenged insufficiently in the enzyme reaction environment, acts as an SOD inhibitor [18].

Conclusions

Increasing chloroplastic Fe-SOD 1 and Cu/Zn-SOD Csd2 isoform amounts may be precise and sensitive biochemical markers of stress conditions that reveal, in particular, the effect of even small doses of aquatic contaminants on green algae.

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