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Copper Toxicity in Leaves of *Elodea canadensis* Michx.

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Abstract *Elodea canadensis* (Canadian waterweed) has an ability to accumulate and bioconcentrate heavy metals. In this work, selected cellular responses for Cu treatment were studied in leaves of *E. canadensis*. Short term experiments, i.e. 1 week exposure to 0.5, 1, 5, and 10 μM of Cu indicated that concentrations up to 10 μM Cu causes a pronounced accumulation of photosynthetic pigments, a drastic degradation of soluble proteins with molecular weight above 18 kDa and a rapid accumulation of polypeptides with molecular weight below 14 kDa. The connection of these observations with copper detoxification mechanisms in aquatic macrophytes are discussed.

Keywords Copper toxicity · Photosynthetic pigments · Copper binding peptides · *Elodea canadensis*

Environmental exposure of aquatic ecosystems to copper is on rising trend owing the use of this metal in a broad spectrum of industrial and domestic applications, particularly in antifouling paints (Ma et al. 2003; Andrade et al. 2004). Copper is an essential nutrient for plant growth and development, and its estimated content is about 10 μg g^{-1} of dry weight of plant tissue. Due to its redox properties, copper is a structural and catalytic component of many proteins and enzymes that are involved in a variety of metabolic pathways (Mal et al. 2002; Marschner 1995; Maksymiec 1997; Yruela 2005). However, Cu ions interfere with numerous physiological processes. They are known to damage cell membranes by binding to the sulphydryl groups of membrane proteins and by increased lipid peroxidation (De Vos et al. 1991, 1993). Copper is an efficient generator of toxic reactive oxygen species (ROS) such as $O_2^{\cdot -}$, H_2O_2, and $HO^{\cdot}$ in Fenton type reactions (Kappus 1986) and is known to induce oxidative stress through the breakdown of polyunsaturated lipids (De Vos et al. 1993). The reactive oxygen species generated by Cu induce severe lipid peroxidation due to the removal of hydrogen from unsaturated fatty acids leading to the formation of lipid radicals and reactive aldehydes. As a consequence, the induced reactions will cause distortion of the lipid bilayer and membrane proteins (Logani and Davies 1980). This process generates large quantities of MDA (malondialdehyde), which is commonly used as an indicator of lipid peroxidation. Additionally, ROS can damage photosynthetic apparatus by directly acting on photosynthetic pigments (Devi and Prasad 1998; Dewez et al. 2005; Vajpayee et al. 2005) and may also catalyse degradation of proteins through oxidative modification and increased proteolytic activity (Romero-Puertas et al. 2002). Copper disrupts the integrity of thylakoid membranes and the constituent fatty acid composition (De Vos et al. 1991), interferes with the biosynthesis of photosynthetic machinery and decreases net photosynthetic rate (Cook et al. 1997; Yruela 2005).

Hydrophytes are extremely important components of water bodies and are vital for primary productivity and nutrient cycling (Nyquist and Greger 2007; Prasad et al.

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2006). Due to a very thin cuticle, submerged hydrophytes can effectively uptake metals directly from the water. In polluted aquatic ecosystems, macrophytes act as biofilters of toxic environmental contaminants and pollutants (Brown and Rattigan 1979; Ribeyre and Boudou 1994). Hydrophytes are reported to accumulate trace metals (thousand to several thousand folds) which otherwise are toxic to biota when present in easily bioavailable form in the interstitial waters (St-Cyr et al. 1994). Metal uptake in hydrophytes depends upon the biosynthesis of metal-binding ligands, particularly thiol-containing compounds and/or the formation of metal chelating peptides/proteins (metalothineins) (Cobbett and Goldsbrough 2002).

Considerable work has been carried out using different species of *Elodea* as model experimental systems for water quality assessment as well as heavy metal accumulation and toxicity (Rabe et al. 1982; Nyquist and Greger 2007). Yet, the information is scanty about the physiological adaptation of *Elodea* to potentially toxic copper concentrations. Hence, to investigate the mechanisms of Cu detoxification in *Elodea*, several cellular responses were researched in short-term experiments (1 week exposure) at environmentally realistic concentrations (0.5, 1, 5, and 10 μM of Cu). We report here the detection of low-molecular weight peptides induced by copper treatment.

Materials and Methods

Elodea canadensis shoots (10–15 cm in length) were collected from the semi-natural population of the Botanical Garden of the Jagiellonian University and maintained under the laboratory conditions (at temperature about 23°C under the daylight) in aquariums with 0.1% Hoaglands nutrient solution, pH 6–7. After 1 week of the incubation in copper-free medium, the experimental material was subjected to treatment with Cu (copper sulfate) in concentrations 0.5, 1.0, 5.0, 10 μM. After 7 days of the incubation the plant material was washed 0.01% Na-EDTA twice with deionized water for the removal of metal adsorbed on the surface of leaves which were used for further analysis. For estimation of a dry weight, plant material was dewatered on tissue paper and the known amount of the fresh weight (usually 2 g) was dried at 55°C for 24 h and subsequently 24 h at 106°C.

For photosynthetic pigment assay, *Elodea* leaves (fresh weight ~20–30 mg) were homogenized on ice with mortar and pestle in 80% of cold acetone. After centrifugation for 10 min at 5,000*g*, the absorbance of pigment extract was measured at wavelengths 470, 625, 646, 664, 730 nm with a Specord M 40 (Zeiss, Jena, Germany) spectrophotometer. The contents of Chl a, Chl b and carotenoids were estimated according to Lichtenthaler (1987).

For an estimation of total soluble thiols and membrane-bound thiols, all isolation steps were carried out at 4°C. The plant material (0.2 g) was homogenized on ice with mortar and pestle in 5 mL of cold Tris–HCl buffer (0.1 M, pH 7.8). The homogenate was centrifuged for 25 min at 15,000*g* in the refrigerated centrifuge at 4°C (centrifugation I) and the supernatant was collected. The pellet was resuspended in 5 mL of cold buffer and centrifuged again in the same conditions (centrifugation II). The supernatants from centrifugations I and II were pooled (total volume ca. 10 mL) and used for the estimation of total soluble proteins and total soluble thiols.

To isolate the membrane-bound fraction, the pellets from centrifugations I and II were pooled and solubilized by stirring with 5 mL of 0.1% triton X-100 in Tris–HCl buffer (0.1 M, pH 7.8) for 15 min. The fraction was clarified by centrifugation (25 min, at 15,000*g*). The pellet was solubilized again with a fresh buffer volume under the same conditions and clarified by centrifugation. The supernatants, containing the membrane-bound fraction, were pooled and used for the estimation of membrane-bound proteins and thiols.

The concentration of thiol groups was estimated according to Ellman (1959) with some modifications. The reaction mixture contained 300 μL of the supernatant, 300 μL 10% SDS, 2.4 mL Tris–HCl buffer (100 mM, pH 7.8), 300 μL of 10 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) and incubated for 1 h at 37°C. After incubation, the absorbance was measured at 412 nm. The reduced glutathione (GSH, Sigma) was used as a standard.

SDS–PAGE: Aliquots of soluble protein fractions were mixed with equal volumes of 62.5 mM Tris–HCl buffer (pH 6.8), containing 10% glycerol (w/v), 5% mercaptoethanol, 10% SDS, and 0.01% bromphenol blue. The samples were boiled for 5 min and equal amounts of protein were loaded onto 18% polyacrylamide gel (Laemmli buffer system) (Ausubel et al. 1995). The gel was resolved at 200 V and proteins were detected by a silver staining according to Blum et al. (1987). Protein concentration was measured according to Bradford (1976), using commercial Bradford reagent (Sigma) and the bovine serum albumin (Sigma) as a standard.

All experiments described in this article were repeated three times. Data presented here are the average means ± standard error. Statistical significance of the results was determined using Student's *t*-test, $p < 0.05$.

Results and Discussion

Elodea plants could grow for 7 days in the presence of Cu in concentrations up to 10 μM in the nutrient medium without any apparently visible necrotic changes. Usually,

after 3–4 days of culture, the chlorosis of leaves became remarkable in plants treated with higher concentrations of Cu (5–10 μM). The content of main photosynthetic pigments (chlorophyll a, b and total carotenoids) was estimated quantitatively in acetone extracts from untreated and copper-treated *Elodea* leaves using absorption coefficients at specific wavelengths given by Lichtenthaler (1987) and recalculated per gram of dry weight. As it is shown in Fig. 1a, the leaves from untreated plants contained ca. twice more chlorophyll a than chlorophyll b. The estimated total amount of carotenoids was lower (ca. 30% in comparison to chl a). The incubation with increasing Cu concentrations resulted in a dose–dependent decrease of pigment content in plant tissues. In particular, the content of chlorophylls has been found to be remarkably sensitive for Cu treatment. In plants treated for 7 days with 10 μM of Cu the chl a content decreased to ca. 23% of values observed in the control material. Total carotenoids were found to be slightly less sensitive for Cu, although the contents of these pigments were also significantly diminished in plants treated with Cu. The comparison of pigment ratios (Fig. 1b) shows that the relative decrease of chlorophyll/carotenoid ratio (up to ca. 75% of control values) observed after treatment with Cu concentrations 1 μM and higher, is caused by a lower accumulation of both chl a and chl b in Cu-treated leaves. The chl a/b ratio was not significantly changed after Cu treatment. Moreover, a slight increase of this parameter was observed in the presence of Cu in concentrations up to 1 μM.

The protein content in the soluble fraction of homogenate from *Elodea* leaves as measured by the Bradford assay, decreased in dose–dependent manner upon Cu treatment. In plants treated with 10 μM of Cu this parameter decreased by ca. 27% in comparison to the control material. On the contrary, the relative concentration of thiol groups in this fraction as measured by Ellman method did not change significantly in leaves treated with different Cu concentrations (Fig. 2a). In the membrane fraction the changes in protein concentration upon Cu exposure were below 10% in comparison to the control material. Also, the relative concentration of thiol groups in this fraction was not drastically altered (Fig. 2b).

To analyze the protein composition, equal amounts of soluble and membrane protein fractions from untreated and copper-treated plants have been subjected to SDS–PAGE. In soluble fraction, the stepwise enhancement of some bands attributable to peptides with apparent MW below 14 kDa was observed in leaves treated with increasing Cu concentrations. Also, the abundance of bands attributable to polypeptides with MW above 18 kDa was seriously

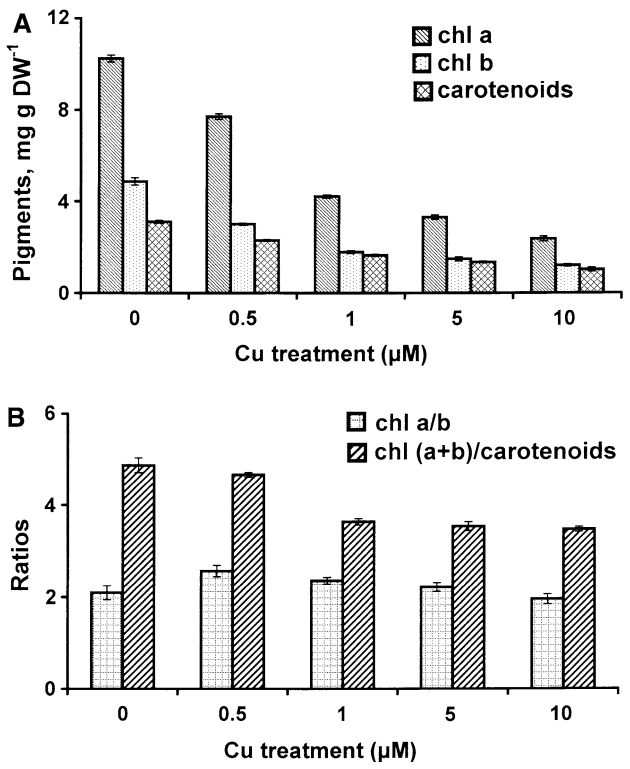

Fig. 1 The changes in concentrations of main photosynthetic pigments (**a**) and in relative pigment ratios (**b**) in *Elodea* leaves treated for 7 days with 0, 0.5, 1.0, 5.0, and 10 μM of Cu

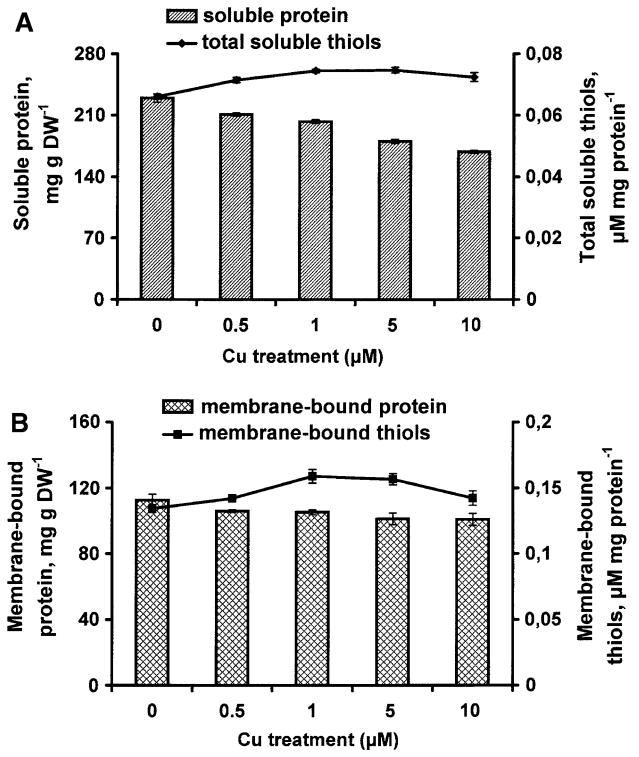

Fig. 2 The changes in concentrations of proteins (*bars, left ordinate*) and total thiols (*solid circles, right ordinate*) in soluble fraction (**a**) and in membrane fraction (**b**) isolated from *Elodea* leaves treated for 7 days with 0, 0.5, 1.0, 5.0, and 10 μM of Cu

lowered in soluble fraction from plants treated with 5 and 10 μM of Cu (Fig. 3a). In contrast, in the membrane fraction, these Cu-enhanced, low MW polypeptides were not detected by SDS–PAGE. Also, the protein composition of this fraction was not significantly altered upon Cu exposure (Fig. 3b).

Elodea canadensis subjected to treatment with Cu at concentrations up to 10 μM for 7 days exhibited pronounced changes in concentrations of photosynthetic pigments. Physiological responses of leaves to Cu exposition, as determined in this study, are summarized and compared in Fig. 4. In particular, concentrations of chl a, chl b and total carotenoids have been found to be significantly reduced in Cu-treated plants. This result suggests that pigment biosynthesis and/or pigment accumulation might be an important target of Cu-induced stress in aquatic macrophytes (Myśliwa-Kurdziel and Strzałka 2002; Myśliwa-Kurdziel et al. 2004). However, low Cu concentrations (up to 1 μM) may stimulate pigment accumulation, as it was previously shown for *Ceratophyllum demersum* and *Lemna trisulca* (Devi and Prasad 1998; Prasad et al. 2001). In our study, this hormetic effect of low doses of Cu was not observed for *E. canadensis*. This leads to the conclusion that the resistance of the photosynthetic apparatus to externally applied Cu might depend on the particular species of aquatic macrophyte. The lowered accumulation of pigments in leaves treated with 5–10 μM of Cu suggests that in our experimental conditions the capacity of photosynthetic apparatus has been diminished. The presence of chlorosis not accompanied with necrotic changes suggests that Cu in concentrations used in our experiments was found to be at the sublethal level. Chlorophyll a and b are the major pigments participating in photosynthesis. Chlorosis is associated with a decreased content of total chlorophyll. The observation that the chl a/b ratio was not significantly changed after Cu treatment suggests that both chl a and chl b are equally sensitive for Cu treatment. The remarkable decrease of total chlorophyll/carotenoid ratio in leaves treated with Cu at concentrations above 1 μM supports the conclusion that the carotenoid pool is more resistant for abiotic stress imposed by Cu and/or carotenoid biosynthesis is elevated upon Cu exposure. The enhanced carotenoid biosynthesis under mild abiotic stress conditions has been observed in other aquatic organisms (Mazurek et al. 1990; Rai et al. 1998).

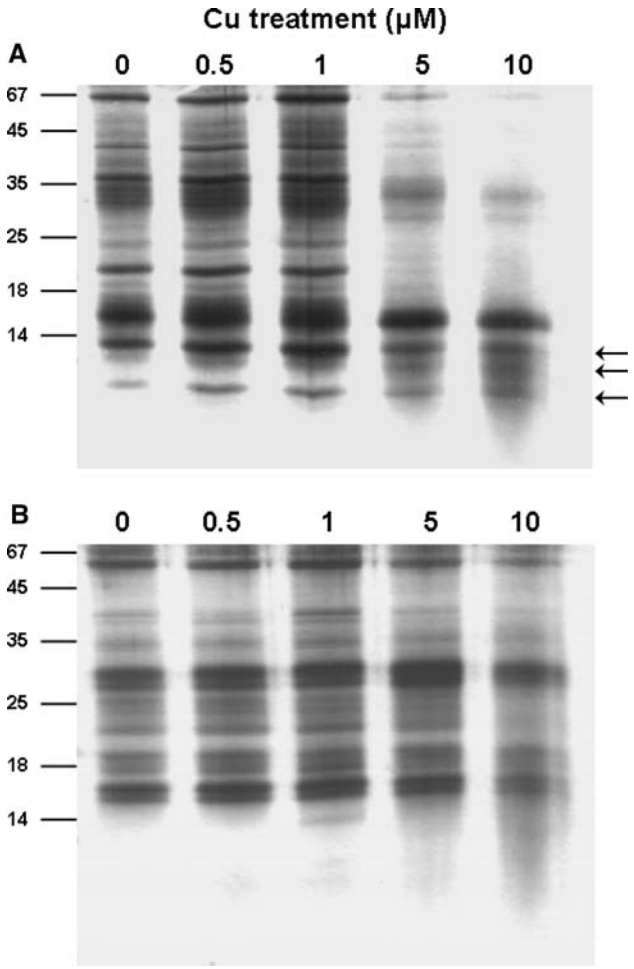

Fig. 3 SDS–PAGE of total soluble protein fraction (**a**) and membrane fraction (**b**) from *Elodea* leaves treated for 7 days with 0, 0.5, 1.0, 5.0, and 10 μM of Cu. The polypeptide bands which appeared or became enhanced in response to Cu are indicated by *arrows*. Silver stained

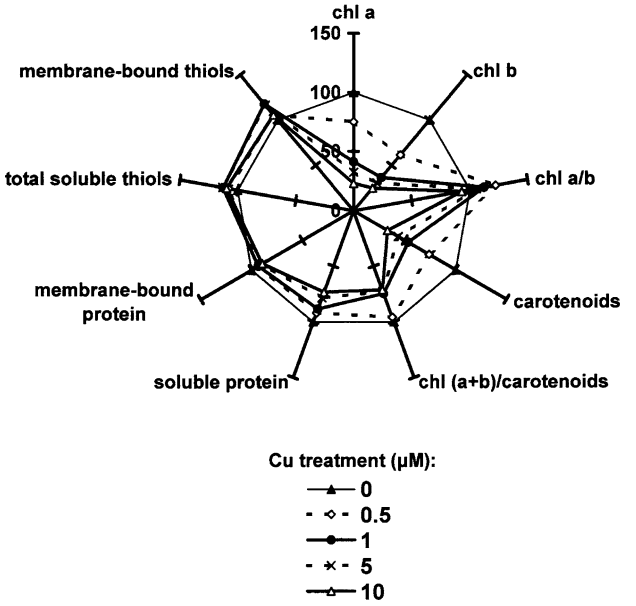

Fig. 4 The *star-diagram* summarizing and correlating normalized values of physiological parameters measured in this study after treatment *Elodea canadensis* with increasing concentrations of copper (0–10 μM). The central point of the *star-diagram* represents '0%' value of each parameter (*solid triangles* represent control values 100%)

In contrast to photosynthetic pigments, the relative concentrations of both soluble and membrane bound proteins in these cellular fractions became less sensitive for Cu treatment. This lack of profound quantitative changes suggests that the protein biosynthesis is not directly affected by elevated concentrations of Cu. The observed decrease of the content of polypeptides with molecular weight above 18 kDa in the soluble protein fraction suggests that externally applied Cu may promote protein degradation in *Elodea* leaves. This process may be an effect of damages caused by enhanced oxidative stress, induced by elevated Cu concentrations (Weckx and Clijsters 1996). On the other hand, the unchanged electrophoretic profiles of membrane protein fractions indicate that the overall membrane structure is not affected by treatment with Cu at concentrations up to 10 μM. Consequently, the integrity of cellular compartments seems to be not altered under our experimental conditions. This result is consistent with our previous findings on another aquatic macrophyte *Lemna trisulca*. In this plant, no apparent protein degradation was observed by SDS–PAGE during Cu treatment at concentrations up to 50 μM (Prasad et al. 2001).

The enhancement of transcription of type 1 and 2 metalothionein genes upon Cu exposure was shown for several terrestrial plant species (Rauser 1999; Cobbett and Goldsbrough 2002). However, limited attention has been given to the identification of plant metalothioneins in situ (Sanita di Toppi et al. 2007). Here, we demonstrate that at least two polypeptides with an apparent molecular weight below 14 kDa show an increased abundance in the soluble fraction from *Elodea* leaves treated with Cu. Although the molecular nature of these polypeptides remains to be elucidated, this result suggests that Cu at micromolar concentrations may induce the expression of specific metalothioneins in shoots of *Elodea*. Surprisingly, the observed accumulation of these putative thiol-enriched polypeptides was not accompanied with an increase of the concentration of reducing thiol groups in Cu-treated leaves (soluble fraction), as measured by the Ellmann's method. This paradox suggests that either most thiol groups synthesized in response to elevated Cu concentrations undergo an immediate saturation with metal and cannot be detected chemically, or, some additional mechanism(s) of effective Cu sequestration may function in leaves of *Elodea canadensis*. Low molecular weight chelators, e.g., organic acids and amino acids are the most likely candidates (Rauser 1999).

In conclusion, our results indicate that Cu, at concentrations up to 10 μM, causes remarkable physiological effects on *Elodea* leaves, including a significant decrease of the accumulation of photosynthetic pigments, a degradation of soluble proteins with molecular weight above 18 kDa and the accumulation of polypeptides with molecular weight below 14 kDa. Further research is needed to elucidate the relations between processes identified in this study and general mechanisms of heavy metal detoxification in aquatic macrophytes.

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