Biometals (2010) 23:315–325
DOI 10.1007/s10534-010-9290-8

Differential responses to cadmium induced oxidative stress in marine macroalga *Ulva lactuca* (Ulvales, Chlorophyta)

Manoj Kumar · Puja Kumari · Vishal Gupta · P. A. Anisha · C. R. K. Reddy · Bhavanath Jha

Received: 7 January 2010 / Accepted: 12 January 2010 / Published online: 30 January 2010

Abstract This study describes various biochemical processes involved in the mitigation of cadmium toxicity in green alga *Ulva lactuca*. The plants when exposed to 0.4 mM $CdCl_2$ for 4 days showed twofold increase in lipoperoxides and H_2O_2 content that collectively decreased the growth and photosynthetic pigments by almost 30% over the control. The activities of antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR) and glutathione peroxidase (GPX) enhanced by twofold to threefold and that of catalase (CAT) diminished. Further, the isoforms of these enzymes, namely, Mn-SOD (~85 kDa), GR (~180 kDa) and GPX (~50 kDa) responded specifically to Cd^{2+} exposure. Moreover, the contents of reduced glutathione (3.01 fold) and ascorbate (1.85 fold) also increased substantially. Lipoxygenase (LOX) activity increased by two fold coupled with the induction of two new isoforms upon Cd^{2+} exposure. Among the polyunsaturated fatty acids, although $n-3$ PUFAs and $n-6$ PUFAs (18:3$n-6$ and C18:2$n-6$) showed relatively higher contents than control, the latter ones showed threefold increase indicating their prominence in controlling the cadmium stress. Both free and bound soluble putrescine increased noticeably without any change in spermidine. In contrast, spermine content reduced to half over control. Among the macronutrients analysed in exposed thalli, the decreased K content was accompanied by higher Na and Mn with no appreciable change in Ca, Mg, Fe and Zn. Induction of antioxidant enzymes and LOX isoforms together with storage of putrescine and $n-6$ PUFAs in cadmium exposed thallus in the present study reveal their potential role in Cd^{2+} induced oxidative stress in *U. lactuca*.

Keywords Antioxidant enzymes · Cadmium · LOX · Minerals · Oxidative stress · PUFAs · *Ulva lactuca*

M. Kumar · P. Kumari · V. Gupta · C. R. K. Reddy (✉) · B. Jha
Discipline of Marine Biotechnology and Ecology, Central Salt and Marine Chemicals Research Institute, Council of Scientific and Industrial Research (CSIR), Bhavnagar 364021, India
e-mail: crk@csmcri.org

P. A. Anisha
School of Environmental Studies, Cochin University of Science and Technology, Cochin, India

Introduction

Of the toxic substances contaminating the aquatic environment, heavy metals particularly cadmium, lead and mercury are of great concern for humans as well as for the environment because of their acute toxicity and high mobility in food chain (Sokolova et al. 2005). Cadmium (Cd^{2+}), with no reported biological function except one occasion as a cofactor for carbonic anhydrase in marine diatom (Lane and

Morel 2000) has been classified as a group (I) carcinogen in humans by the International Agency for Research on Cancer (IARC 1993). Cd^{2+} being an oxophilic and sulfophilic element forms complexes with various organic particles and thereby triggers a wide range of reactions that collectively make the aquatic ecosystem at risk (Webster et al. 1997). The Cd^{2+} even at trace concentration disturbs the cellular metabolic process by producing excessive reactive oxygen species (ROS) leading to oxidative stress.

Acclimation of seaweeds to heavy metal induced oxidative stress involves a complex enzymatic and non-enzymatic antioxidant system that functions in a more coordinated manner to mitigate the cellular osmolarity, ion disequilibrium and detoxification of ROS (Collen et al. 2003; Malea et al. 2006; Ratkevicius et al. 2003; Wu and Lee 2008). However, the involvement of antioxidants in response to Cd^{2+} induced stress in macroalgae is unclear, because it is not a transition metal like Cu and Fe, which may induce oxidative stress via a Fenton-type reaction. Although ROS is commonly known to react with proteins, nucleic acids and lipids causing deleterious effects on various cellular processes, it also generates oxygenated polyunsaturated fatty acids (Ox-PUFAs) defending the oxidative stress. A great deal of information supporting the involvement of Ox-PUFAs in abiotic and biotic stresses has also recently implicated the function of lipoxygenase (LOX) enzyme in the stress physiology (Maksymiec and Krupa 2006; Rucinska and Gwozdz 2005). Ritter et al. (2008) also reported the synthesis of octadecanoid and eicosanoid oxygenated derivatives in *Laminaria digitata* following the exposure to Cu stress. Nevertheless, the involvement of LOX and the differential induction of its isoforms have largely been remained as unexplored in seaweeds under Cd^{2+} stress.

Although the mode of Cd^{2+} action is largely unknown, its high affinity for sulfhydryl and oxygen containing groups results in blocking the essential functional groups of biomolecules (Webster et al. 1997). Consequently, it inhibits the uptake and transport of many macro/micronutrients and thus, induces the nutrient deficiencies. Further, polyamine (PAs)—aliphatic amines with relatively low molecular mass have also been studied in macroalgae with respect to their involvement in cell division (Cohen et al. 1984; Garcia Jimenez et al. 1998) and protection from hypo saline stress (Lee 1998; Garcia Jimenez et al. 2007). Sacramento et al. (2004, 2007) and Guzman-Uriostegui et al. (2002) have reported their role in maturation of reproductive structure in *Grateloupia* and *Gracilaria cornea* respectively, but their function as metal chelator to protect the seaweeds from metal induced oxidative stress has not been reported.

In the present study, toxicology of cadmium was determined using a green alga *Ulva lactuca*, a known bioindicator of heavy metal pollution (Ho 1990). The growth patterns, lipid peroxidation, and H_2O_2 content were quantified as an indication of cellular damage induced by exposure to cadmium. Subsequently, the regulation of antioxidant enzymes, polyamines, lipoxygenase, photosynthetic pigments, polyunsaturated fatty acids and nutrient imbalance was determined to evaluate their possible role in combating the cadmium toxicity. This is the first time changes in the isoforms of major antioxidative and lipoxygenase enzymes and bioaccumulation of polyamines were examined as a function of Cd^{2+} induced oxidative stress in *U. lactuca*.

Materials and methods

Algal culture and $CdCl_2$ treatment

Ulva lactuca was collected from Veraval Coast (20°54′ N, 70°22′ E), Gujarat, India during March 2009. Selected clean and healthy thalli were carried in a cool pack to the laboratory. In order to initiate unialgal culture, the rhizoidal portions were removed to eliminate contaminants and then the fronds were cleaned manually with brush in autoclaved seawater to remove epiphytic foreign matters. The fronds thus cleaned were acclimatized to laboratory conditions by culturing in aerated flat bottom round flasks in PES medium (Provasoli 1968) supplemented with GeO_2 (5 mg L^{-1}) for 10 days. During the acclimatization period, the medium was replenished every alternate day and maintained under white cool fluorescent tube lights at 50 μmol photons m^{-2} s^{-1} with a 12:12 h light:dark cycle at 22 ± 1°C. Following the acclimatization period, healthy thalli (0.2 g FW) were cultured in autoclaved natural seawater (1:2 w/v) supplemented with different concentration of Cd^{2+} ranging from 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 mM for 4 days without adding any nutrient and chelators during the experiment while keeping other

conditions similar to the acclimatization period described earlier. There were three replicates for each metal concentration.

Determination of growth, lipoperoxides, H_2O_2 and total protein content

After blotting the algae with paper towels daily growth rate (DGR) was measured as increase in fresh weight (FW) after 4 days and calculated by using formula $DGR\% = [(W_4/W_o)^{1/4}-1] \times 100$, where W_4 represents fresh weigh after 4 days and W_o as initial fresh weight. The level of lipid peroxidation in the thallus was determined by the thiobarbituric acid reacting substances (TBARS) resulting from the thiobarbituric acid (TBA) reaction as described by Heath and Packer (1968). The concentration of TBARS was calculated by subtracting the non specific absorbance measured at A_{600} from A_{532} (ε-155 mM^{-1} cm^{-1}). Hydrogen peroxide was measured by homogenizing the tissue in an ice bath with 0.1% (w/v) trichloroacetic acid (TCA) (Lee and Shin 2003). The supernatant after centrifugation was mixed with 50 mM potassium phosphate buffer (pH 7.0) and 1 M KI. The absorbance of the supernatant was read at 390 nm and H_2O_2 content was obtained from a standard curve for H_2O_2. Total proteins were extracted by homogenizing 0.2 g FW in 1 mL of extraction buffer containing 0.5 M Tris–HCl (pH 8.0), 0.7 M sucrose, 50 mM ethylenediaminetetraacetic acid (EDTA), 0.1 M KCl, 2% (v/v) β-mercaptoethanol and 2 mM phenylmethylsulfonyl fluoride under cool conditions. The homogenates were centrifuged at 12,000 g for 20 min at 4°C. An aliquot of 100 µL of the supernatant was used for protein estimation with the method described by Bradford (1976).

Determination of pigments, aminolevulinic acid dehydratase, non enzymatic antioxidants, polyamines and minerals

The photosynthetic pigments chlorophyll *a*, *b* and carotenoids were extracted in 80% acetone by homogenizing the tissue in ratio (1:4 w/v). The amount of these pigments was calculated using the formula for Chl $a = 11.75\ A_{662} - 2.350\ A_{645}$; for Chl $b = 18.61\ A_{645} - 3.960\ A_{662}$ and for Carotenoids = (1,000 $A_{470} - 2.270$ Chl $a - 81.4$ Chl *b*)/227, formulated by Lichtenthaler and Wellburn (1985). Extract for the determination of aminolevulinic acid dehydratase (ALA-D) was prepared in 100 mM Tris–HCl buffer (pH 9.0) containing 0.1% Triton X-100 and 0.5 mM dithiotreithol (DTT) at a proportion of 1:1 (w/v). ALA-D activity was assayed as described by Morsch et al. (2002) measuring the rate of porphobilinogen (PBG) formation. The concentration of total ascorbate [reduced ascorbate (AsA) + oxidized ascorbate (DHA)] and total glutathione [reduced glutathione (GSH) + oxidized glutathione (GSSG)] were determined according to the procedures described by Wu and Lee (2008). Oxidized AsA (DHA) content was calculated by the subtraction of AsA from total AsA content. The reduced GSH content was calculated by the subtraction of oxidized GSH content from total glutathione content. Polyamines were extracted from 500 mg fresh tissue and estimated following the method described by Guzman-Uriostegui et al. (2002). Accumulation of Cd^{2+} and contents of macro and micro nutrients were determined in control and Cd^{2+} treated plants dried to constant weight at 60°C. Dried tissues were acid digested by $HNO_3/HClO_4$ (5:1, v/v) and then analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Perkin–Elmer, Optima 2000).

Determination of antioxidative enzymes and lipoxygenase

Extracts for determination of superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPX), catalase (CAT) and ascorbate peroxidase (APX) activities were prepared under ice-cold conditions in the respective extraction buffer at a proportion of 1:2 (w/v) as described by Wu and Lee (2008). The SOD activity was determined by photochemical inhibition of nitro blue tetrazolium (NBT). The CAT activity was measured at A_{420} nm for H_2O_2 decomposition rate using the extinction coefficient of 40 mM^{-1} cm^{-1}. Total APX activity was measured by monitoring the decline in A_{290} for 3 min as ascorbate oxidized (ε-2.8 mM^{-1} cm^{-1}). The GR activity was determined by monitoring the decline in A_{340} for 5 min as NADPH oxidized (ε-6.2 mM^{-1} cm^{-1}). One unit of enzyme activity is defined as 1 µmol min^{-1} for CAT, APX, GR and GPX, while one unit of SOD is defined as the 50% inhibition of activity of the control (without sample extract added). Extract for lipoxygenase (LOX) was prepared according to the method described by Tsai et al. (2008) and assayed by

measuring the increase in absorbance at 234 nm with substrate linolenic acid (100 μM) prepared in ethanol. LOX activity was determined using extinction coefficient 25,000 L mol^{-1} cm^{-1}. The isoenzyme profiles of antioxidative enzymes and lipoxygenase were determined on 10 or 12% non-denaturating polyacrylamide gels using their specific activity staining procedures for SOD (Beauchami and Fridovich 1971), APX (Mittler and Zilinskas 1994), GR (Rao et al. 1996), GPX (Lin et al. 2002) and LOX (Heinisch et al. 1996). The molecular mass of enzyme isoforms was evaluated comparing with the standard molecular weight marker containing myosin, 210 kDa; β-galactosidase, 135 kDa; bovine serum albumin, 80 kDa; soyabean trypsin inhibitor, 31.5 kDa and lysozyme, 18.2 kDa.

Extraction and analysis of fatty acids

Fatty acids from lipids were converted to respective methyl esters by trans-methylation using 1% NaOH in methanol and heated for 15 min at 55°C, followed by the addition of 5% methanolic HCl and again heated for 15 min at 55°C. Fatty acid methyl esters (FAMEs) were extracted in hexane and analyzed by GC-2010 coupled with GCMS-QP2010.

Statistics

All data presented were means ± standard deviation of three independent experiments. Statistical analyses were performed by one way analysis of variance (ANOVA). Significant differences between means were tested by the least significant difference (LSD) at 0.01 and 0.05 probability levels.

Results

Growth rate, contents of TBARS, H_2O_2 and total protein

Addition of $CdCl_2$ to the culture medium for 4 days significantly decreased the daily growth rate (DGR) of *U. lactuca* in a dose dependent manner (Table 1). At concentration 0.4 and 0.5 mM Cd^{2+} the growth rate reduced markedly by almost 30 and 48.72% respectively, compared to that of control with 4.29% DGR. The Cd^{2+} content in treated thallus also increased linearly with the metal concentration and accumulated significantly (>five fold) in thallus grown at ≥0.5 mM Cd^{2+} (Table 1). Increased lipoperoxides (TBARS content) and H_2O_2 content as oxidative stress biomarkers significantly reduce the total protein contents in Cd^{2+} treated thallus. As compared to control, the content of both TBARS and H_2O_2 increased by ≥two fold in thallus exposed to 0.4 mM or higher Cd^{2+} concentrations. The total protein content decreased by 30.71% ($P < 0.01$) at 0.4 mM Cd^{2+}. This decrease was more prominent in thallus exposed to higher Cd^{2+} concentration (>0.5 mM) which eventually led to severe chlorosis, loss of thallus rigidity and reduced biomass. As a result, 0.4 mM Cd^{2+} was chosen as extreme concentration for the subsequent experiments in order to

Table 1 Data on growth, MDA, H_2O_2 and protein content of *U. lactuca* following its exposure to $CdCl_2$ for 4 days (mean of three independent experiments ± SD)

$CdCl_2$ (mM)	DGR (%)	TBARS (nmol g^{-1} FW)	H_2O_2 (μmol g^{-1} FW)	Total Protein (mg g^{-1} FW)	Cd uptake (μg g^{-1} DW)
Control	4.29 ± 0.32^{a}	4.11 ± 0.58^{d}	0.19 ± 0.03^{f}	8.24 ± 0.50^{a}	0.17 ± 0.03^{d}
0.1	4.01 ± 0.15^{ab}	6.10 ± 0.83^{c}	0.24 ± 0.04^{ef}	7.92 ± 0.18^{ab}	0.22 ± 0.04^{d}
0.2	3.75 ± 0.32^{ab}	7.95 ± 0.31^{bc}	0.35 ± 0.02^{de}	7.17 ± 0.92^{ab}	0.26 ± 0.03^{d}
0.3	3.43 ± 0.18^{bc}	9.26 ± 1.35^{b}	0.41 ± 0.05^{d}	6.58 ± 0.71^{bc}	0.45 ± 0.04^{c}
0.4	3.05 ± 0.30^{c}	11.33 ± 1.67^{a}	0.47 ± 0.06^{d}	6.33 ± 0.40^{c}	0.56 ± 0.06^{c}
0.5	2.23 ± 0.35^{d}	15.49 ± 1.38^{a}	0.64 ± 0.05^{c}	4.47 ± 0.87^{d}	0.96 ± 0.08^{b}
0.6	1.08 ± 0.25^{e}	16.30 ± 1.69^{a}	0.79 ± 0.06^{b}	3.78 ± 0.31^{de}	1.07 ± 0.04^{ab}
0.7	-0.64 ± 0.12^{e}	18.76 ± 2.89^{a}	1.04 ± 0.12^{a}	2.56 ± 0.43^{e}	1.16 ± 0.08^{a}
LSD (1%)	0.62	3.66	0.14	1.42	0.13

Different superscript letters within column indicates significant differences at $P < 0.01$ according to one way ANOVA

minimize excessive toxicity and cell death as a result of extreme concentrations.

Changes in photosynthetic pigments, aminolevulinic acid dehydratase and antioxidants

The content of chl *a*, *b* and carotenoid got significantly affected with Cd^{2+} treatment (Table 2). The content of chl *a* and *b* in Cd^{2+} treated thallus reduced significantly by 32.82 ($P < 0.05$) and 23.36% ($P < 0.05$) from the control with 115.56 ± 4.11 and 62.66 ± 5.04 μg g^{-1} FW respectively. However, the carotenoid content was stable when compared with control value 24.17 ± 2.52 μg g^{-1} FW. Thus, carotenoid/total chlorophyll ratio increased to 0.25 ($P < 0.05$) in the treated thallus over the control

Table 2 Effect of Cd^{2+} on photosynthetic pigments, ALA-D enzyme activity, antioxidants, polyamines and nutrient imbalance in *U. lactuca* (mean of three independent experiments ± SD)

Parameter	Cd^{2+} (mM)		LSD (5%)
	Control	0.4	
Photosynthetic pigments and enzyme			
Chl *a* (μg g^{-1} FW)	115.56 ± 7.18[a]	77.64 ± 5.66[b]	14.68
Chl *b* (μg g^{-1} FW)	62.66 ± 5.04[a]	48.02 ± 4.49[b]	10.83
Chl *a* + *b* (μg g^{-1} FW)	178.22 ± 8.67[a]	125.66 ± 6.66[b]	11.52
Carotenoid (μg g^{-1} FW)	24.17 ± 2.52[a]	31.44 ± 3.78[a]	7.30
Carotenoid/Chl *a* + *b*	0.14 ± 0.01[b]	0.25 ± 0.04[a]	0.07
Aminolevulinic acid dehydratase (U mg^{-1} protein)	0.36 ± 0.04[a]	0.20 ± 0.03[b]	0.11
Antioxidants (nmol g^{-1} FW)			
(AsA)	0.69 ± 0.05[b]	1.28 ± 0.07[a]	0.14
(DHA)	0.74 ± 0.08[b]	1.00 ± 0.13[a]	0.19
(AsA + DHA)	1.43 ± 0.13[b]	2.28 ± 0.20[a]	0.32
AsA/DHA	0.93 ± 0.05[b]	1.29 ± 0.10[a]	0.18
GSH	1.73 ± 0.26[b]	5.22 ± 0.40[a]	0.76
GSSG	1.16 ± 0.24[b]	2.15 ± 0.22[a]	0.53
GSH + GSSG	2.89 ± 0.49[b]	7.36 ± 0.54[a]	1.18
GSH/GSSG	1.51 ± 0.11[b]	2.44 ± 0.17[a]	0.40
Polyamines (μmol g^{-1} FW)			
Putrescine			
F	8.24 ± 1.27[b]	15.38 ± 3.18[a]	5.48
BS	3.83 ± 1.02[b]	6.53 ± 1.04[a]	2.34
Spermidine			
F	1.86 ± 0.34[a]	2.16 ± 0.29[a]	1.04
BS	0.88 ± 0.17[a]	0.95 ± 0.19[a]	0.41
Spermine			
F	0.87 ± 0.23[a]	0.40 ± 0.09[b]	0.39
BS	0.52 ± 0.06[a]	0.26 ± 0.04[b]	0.18
Macro elements (%DW)			
Na	6.83 ± 1.15[b]	10.51 ± 1.12[a]	2.58
K	3.89 ± 0.40[b]	2.69 ± 0.62[a]	0.88
Ca	0.76 ± 0.10[a]	0.67 ± 0.56[a]	0.25
Mg	0.52 ± 0.06[a]	0.46 ± 0.04[a]	0.11
Trace elements (mg 100 g^{-1} DW)			
Fe	10.96 ± 1.58[a]	7.77 ± 1.61[a]	3.61
Mn	2.53 ± 0.42[b]	3.81 ± 0.39[a]	0.86
Zn	3.32 ± 0.43[a]	2.76 ± 0.34[a]	0.84

Different superscript letters within row indicate significant differences at $P < 0.05$ according to one way ANOVA

F Free, *BS* bound soluble

(0.14). Further, to verify the effect of Cd^{2+} treatment on heme biosynthesis in thallus, the activity of ALA-D enzyme was measured with almost 45% ($P < 0.05$) reduction in metal treated thallus compared to control with 0.36 U mg^{-1} protein.

The contents of total (AsA+DHA), reduced (AsA) and oxidized (DHA) ascorbate were greatly influenced by Cd^{2+} exposure. Their contents significantly increased ($P < 0.05$) by 58.59, 85.99 and 34.01% over control with corresponding values 1.44 ± 0.13, 0.69 ± 0.05 and 0.74 ± 0.08 mmol g^{-1} FW, respectively (Table 2). To determine the regeneration of AsA due to metal exposure, the ratio of AsA/DHA was calculated and found as 1.29 over the control (0.93). Total (GSSG+GSH), reduced (GSH) and oxidized (GSSG) glutathione content in Cd^{2+} treated thallus increased markedly ($P < 0.05$) with 2.55, 3.01 and 1.86 fold respectively over control. The ratio of GSH/GSSG also increased from 1.51 (control) to 2.44 in treated thallus (Table 2).

Changes in the endogenous polyamines contents and nutrient imbalance

The content of the three polyamines (PAs) namely putrescine (Put), spermidine (Spd) and spermine (Spm) were changed significantly due to cadmium treatment (Table 2). The content of both free (F) and bound soluble (BS) Put increased noticeably ($P < 0.05$) by 86.52 and 70.76% respectively. Spermidine content (F and BS) in both control and treated ones measured the same, while Spm reduced to 49% (F) and 42.17% (BS) respectively over control values. Thallus cultured in seawater supplemented with Cd^{2+} for 4 days showed significant variations in nutrient contents (Table 2). The analysis of cadmium treated thallus for macronutrients showed a significant decrease in K content with a parallel increase in Na ($P < 0.05$) content while no noticeable change was observed in Ca and Mg ($P < 0.05$) contents as compared to control. Among the micronutrients, Mn increased significantly ($P < 0.05$), while Fe and Zn did not differ from the control ($P < 0.05$).

Changes in the activities of antioxidative enzymes and lipoxygenase

SOD, CAT, APX, GR and GPX were selected as biomarkers to determine the oxidative stress caused by cadmium on the enzymatic defense system. Exposure to cadmium increased the specific activities of SOD and APX markedly by 2.05 ($P < 0.01$) and 1.62 folds ($P < 0.05$) in the treated thallus over the control activities with 139.33 ± 12.66 and 0.17 ± 0.03 U mg^{-1} protein respectively. However, the increase in GR and GPX activities were more pronounced in the treated thallus with 3.38 and 2.42 fold, respectively ($P < 0.01$) compared to that of control with 0.31 ± 0.08 and 0.60 ± 0.08 U mg^{-1} protein. On the other hand, the specific activity of CAT decreased significantly ($P < 0.05$) by 43.95% in Cd^{2+} treated thallus. In order to ascertain the involvement of LOX in the peroxidation process, we measured its activity in both control and treated thallus. Cd^{2+} treatment markedly enhanced LOX activity (>two fold) compared with that of control (6.3 nkatal min^{-1} protein).

The changes in isoforms of different antioxidative enzymes and LOX (above the column bars) in thallus treated with Cd^{2+} are shown in Fig 1. In the control one Fe-SOD (SOD-1, ~70 kDa) and two Zn-SOD (SOD 3 and 4, ~20 and 35 kDa) isoforms were observed, while one more isoform Mn-SOD (SOD 1, ~85 kDa) was observed in Cd^{2+} treated thallus and was confirmed as by using H_2O_2/KCN as inhibitor. Two isoforms of GR (GR-1 and GR-2) were visualized on 10% activity staining gel in both control and Cd^{2+} treated thallus with approximate molecular weight ranging from 180 to 135 kDa (Fig. 1). However, the levels of activity for GR-1 increased markedly upon metal exposure as compared to the control. The activity gel of GPX showed only single isoform (GPX-1) of nearly 80 kDa in control while an additional isoform GPX-2 with approximate molecular weight 50 kDa was observed in Cd^{2+} treated thallus. A slight increase in the intensity of APX-1 and 2 was also observed in Cd^{2+} treated thallus when compared to control. Further, Cd^{2+} treatment induced two new isoforms of LOX with molecular weight of nearly 80 and 55 kDa in addition to isoforms of 125 kDa recorded in control. However, an isoform of LOX (110 kDa) was completely absent in Cd^{2+} treated thallus though it was prominent in control.

Changes in fatty acid composition

Table 3 shows the variations in fatty acid composition in response to Cd^{2+} stress (0.4 mM). The control contained mostly C16:0, C16-1 ($n - 7$) and C18-1

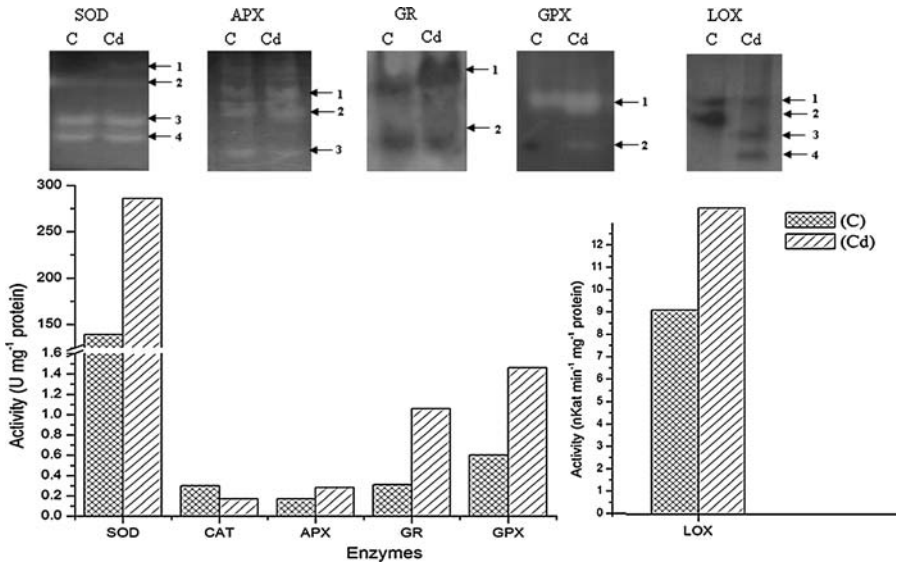

Fig. 1 Antioxidant enzymes activities in *Ulva lactuca* in response to cadmium exposure for 4 days. Cross and non-cross *shaded columns* represents the activities for control (C) and 0.4 mM cadmium chloride (Cd^{2+}) metal exposure respectively for 4 days. *Pictures* above the *columns bars* represent the activity staining gels for SOD, APX, GR, GPX and LOX enzymes extracted from control (C) and metal exposed thalli (Cd^{2+})

($n-9$) acids which together constitute about 65% of the total fatty acid. Exposure of thalli to cadmium supplemented culture medium resulted a decrease in their content by nearly 26% ($P < 0.05$), while a parallel increase was observed for C18:2 ($n-6$) and C18:3 ($n-6$) by ≥two fold compared to their content in control with 3.77% and 3.07% of total fatty acid. This increase contributed significantly for the higher amount of total C18 PUFAs in metal exposed thalli. The content of total C20 PUFAs also showed a significant increase ($P < 0.05$) from 3.96% (control) to 5.65% (Cd^{2+} treatment). Interestingly, the increases for $n-6$ PUFAs were more evident as compared to $n-3$ PUFAs on metal exposure (Table 3).

Discussion

The decreased growth rate with corresponding increase in lipid peroxidation (TBARS content) and

Table 3 Effect of Cd^{2+} on fatty acid composition (% of total fatty acid) in *U. lactuca* (mean of three independent experiments ± SD)

Fatty acids	Cd^{2+} (mM)		LSD (5%)
	Control	0.4	
C14:0	3.46 ± 0.31[a]	3.13 ± 0.28[a]	0.67
C16:0	37.33 ± 0.42[a]	33.53 ± 2.16[b]	3.53
C18:0	3.01 ± 0.09[b]	6.64 ± 0.92[a]	1.48
C20:0	1.18 ± 0.04[a]	1.05 ± 0.07[a]	0.14
C16:1$n-7$	9.64 ± 0.29[a]	5.53 ± 0.53[b]	0.96
C18:1$n-9$	18.73 ± 0.30[a]	12.41 ± 1.19[b]	1.97
C18:2$n-6$	3.77 ± 0.10[b]	8.13 ± 1.04[a]	1.67
C18:3$n-6$	3.05 ± 0.05[b]	6.15 ± 1.71[a]	2.25
C18:3$n-3$	2.92 ± 0.07[a]	3.56 ± 0.20[b]	0.35
C20:4$n-6$	1.94 ± 0.08[a]	2.71 ± 0.67[a]	1.09
C20:5$n-3$	2.02 ± 0.07[b]	2.93 ± 0.40[a]	0.65
C22:6$n-3$	4.33 ± 0.42[a]	5.13 ± 1.06[a]	1.83
Others	8.64 ± 0.12[a]	9.43 ± 0.53[a]	0.88
C18:2$n-6$/C18:1$n-9$	0.20[b]	0.66[a]	0.22
C18:3$n-3$/C18:2$n-6$	0.77[a]	0.44[b]	0.12
C18:3$n-6$/C18:2$n-6$	0.81[a]	0.74[a]	0.21

Others include C12:0, C13:0, C15:0, C17:0, C22:0, C17:1 & C20:1

Different superscript letters within row indicate significant differences at $P < 0.05$ according to one way ANOVA

H_2O_2 content in *Ulva lactuca* following the exposure to cadmium in the present study clearly indicates the ROS generation confirming the state of oxidative stress. The degeneration of chlorophyll leading to decreased photosynthetic activity has been a common response in plants exposed to heavy metals. A noticeable decrease in chlorophyll content ($a + b$) in Cd^{2+} exposed thallus positively correlated with decreased activity of ALA-D enzyme. This enzyme catalyzes the reaction of tetrapyrrol biosynthesis, including chlorophyll molecules and is, therefore, crucial for the sustenance of cellular life. Cadmium perhaps inhibited the ALA-D enzyme activity by interacting with its functional –SH groups eventually interfering with the heme biosynthesis and subsequent chlorophyll formation (Noriega et al. 2007). Further, ALA, a substrate for ALA-D catalyzed reaction, could be another source of generating the superoxide, hydrogen peroxide and hydroxyl radical, if it undergoes enolization and metal-catalyzed aerobic oxidation at physiological pH. Therefore, ALA-D inhibition may lead to ALA accumulation which in turn contributes to enhanced level of ROS in cell (Noriega et al. 2007). The decreased chlorophyll content with increased carotenoids in the metal treated thallus accounted for higher carotenoid/chlorophyll ratio in this study. Therefore, it suggests the role of carotenoids as an antioxidant by acting as physical quenchers of electronically excited molecules, in addition to functioning as photoreceptors (Woodall et al. 1997).

Significant accumulation of both di and tri unsaturated (C18:2$n-6$, linoleic and C18:3$n-6$, linolenic) fatty acids at the expense of dominant saturated (C16:0) and monounsaturated fatty acids (C16:1, C18:1) indicates the induction of desaturation process of fatty acids during cadmium stress at the studied concentration. A threefold increase in the ratio of C18:2$n-6$/C18:1$n-9$ and 1.5 fold increase of C18:3$n-6$/C18:2$n-6$ indicates the induction of $\Delta 12$ and $\Delta 6$ desaturase respectively. At the same time 0.57 folds decrease in C18:3$n-3$/C18:2$n-6$ ratio signifies greater importance of $n-6$ PUFAs over $n-3$ PUFAs in cadmium stress. Free fatty acids like C18:2$n-6$, C18:3$n-6$, C20:5$n-3$ and hydroxylated derivatives have also been shown to involve in defense reactions against the methyl jasmonate mediated oxidative burst in brown algae (Kupper et al. 2009). Chaffai et al. (2007) also implicated enhanced desaturase activity in maize seedlings exposed to Cu stress. This high level of unsaturation of lipids could be required to maintain the degree of fluidity needed for the diffusion of lipophilic compounds, to confer a suitable geometry to the lipid molecules and the activities of membrane-bound enzymes as well (Quartacci et al. 2001).

Moreover, increased activity of lipoxygenase together with the induction of two new isoforms could positively be correlated with the increased $n-6$ PUFAs particularly C18:3$n-6$ and C18:2$n-6$ fatty acids. These two fatty acids are the two main substrates of LOX which catalyzes their oxidation and convert them to either 9- or 13- hydroxyperoxides, or a mixture of both, depending on the enzyme isoforms (Tamas et al. 2008). However, further experiments are needed to establish the nature of primary and secondary products formed by this enzyme in the presence of Cd^{2+} ions. In this context, linoleic/linolenic acid dependent LOX activity and arachidonic acid dependent LOX activity have been reported recently in *Laminaria digitata* (Bouarab et al. 2004) and *Chondrus crispus* (Ritter et al. 2008). Higher LOX activity has been positively correlated with increased lipoperoxides in higher plants such as barley and lupine under cadmium stress (Rucinska and Gwozdz 2005; Tamas et al. 2008). In this context, a number of studies have reported accumulation of lipoperoxides and enhanced ROS production in seaweeds (Burrit et al. 2002; Contreras et al. 2005) but none correlated their accumulation with toxic effects. The higher lipoperoxide level observed in this study is not solely due to higher ROS but could also be due to higher LOX activity. In contrast, lipoperoxides accumulation has been exclusively ROS-dependent in cadmium stresses tobacco plants. Further, LOX generated ROS activation may also occur following strong Cd^{2+} induced ROS production through the activation of NADPH oxidase, oxalate oxidase or oxidative cycle of peroxidases (Zhao and Yang 2008). Most recently, Contreras et al. (2009) described the induction of an arachidonic acid-dependent LOX activity and its role in lipoperoxides production in *Lessonia nigrecens* and *Scytosiphon lomentaria* under copper exposure.

One of the mechanisms that was involved in the prevention of heavy metal induced cell destruction has been the synthesis of antioxidative enzymes (Collen et al. 2003; Ratkevicius et al. 2003; Wu and Lee 2008). Elevated level of antioxidative enzymes

predominantly SOD, GR and GPX in thallus following the Cd treatment in this study demonstrate that these enzymes act in combination to reduce the impact of Cd toxicity. At the same time it is worth noting that the studied Cd concentration (0.4 mM) strongly inhibited the CAT activity thus invariably suggests its sensitivity against O_2^- radicals or peroxisomal proteases. SOD activity is crucial to dismutate the reactive O_2^- ions to H_2O_2 and O_2. Enhanced SOD activity observed in Cd treated thallus could be related to the Mn-SOD isoforms induced in addition to prevailing Fe and Zn-SOD isoforms indicating that it scavenges O_2^- radicals more efficiently. Similar increase in SOD activity has also been reported in *Nannochloropsis oculata* (Lee and Shin 2003) and *Gracilaria tenuistipitata* (Collen et al. 2003) following their exposure to cadmium. Recently, microarray and proteomics studies have also established the transient up-regulation of antioxidant enzyme families such as SOD, GPX and CAT in green algae under copper (Wu and Lee 2008) and cadmium exposure (Smeets et al. 2008).

Apparently, the decreased activity of CAT in the present study was compensated by increased activity of APX, GR and GPX during the Cd^{2+} stress. APX activity appears to be significant and could be attributed to increased activity of APX-1 and APX-2 during Cd^{2+} stress. It is evident from this study that APX is more efficient than catalase in destroying the H_2O_2. The reason for this could be that unlike catalase which is localized to peroxisome only has low substrate affinity. In contrast, APX is present through out the cell and has higher substrate affinity in the presence of AsA as a reductant (Willekens et al. 1995). Increased AsA and GSH pool and their regeneration rate in Cd^{2+} treated thallus suggests their role in detoxifying the H_2O_2 inspite of its accumulation at the studied concentration. AsA is known for its multiple functions apart from being a substrate for APX. It reacts directly with hydroxyl, superoxide radicals, singlet oxygen and H_2O_2 and can also regenerate the lipophilic antioxidant α-tocopherol (Smirnoff 1996). Further, AsA involves in the regulation of photosynthesis and in preserving the activities of enzymes that contain prosthetic transition metal ions (Smirnoff 1996). The extent of increase in GR and GPX activities in Cd^{2+} treated thallus is attributed chiefly to isoforms GR-1 and GPX-1 respectively (Fig. 1). Their increased activity invariably indicates the tolerance strategy of *U. lactuca*, following the Cd^{2+} exposure. As at one hand, a threefold higher content of GSH was maintained via GR while at other hand it was used to detoxifying the H_2O_2 via GPX activity. Malea et al. (2006) reported a fivefold increase in total glutathione pool in *Enteromorpha linza* and ascribed it to Cd^{2+} induced oxidative stress. The higher level of GSH during Cd^{2+} stress is crucial considering that it is the monomeric substrate of phytochelatin that can form complexes with cadmium and sequester it into the vacuoles (Groppa et al. 2007). Moreover, GSH also participates in the regeneration of AsA via dehydroascorbate reductase and can also react with singlet oxygen, OH radical and can guard protein groups (Noctor et al. 2002).

It has been reported in some multicellular marine green algae that PAs, especially Put and Spd, are accumulated under extreme hyposaline conditions (Garcia Jimenez et al. 2007). In the present study, for the first time we have reported the variations in the endogenous level of PAs in seaweed under heavy metal stress like cadmium. In the pathway of polyamine metabolism, adenine or ornithine decarboxylase (ADC or ODC) catalyzes L-arginine/ornithine decarboxylation to form Put, and diamine amine oxidase catalyzes Put to decompose. A twofold increase for Put (both free and bound soluble) with no change in Spm in Cd^{2+} exposed thalli could be attributed due to (1) inhibition of diamine oxidase, (2) induction of polyamine oxidase and (3) increased ethylene formation due to an increased SAM flux. Consequently there was an inhibition in conversion of Put to Spd or Spm, despite Put availability. PAs have been suggested as a potential antioxidant due to strong bindings with anion and cation at physiological pHs. The binding of PAs to anions (phospholipids membranes, nucleic acids) contributes to its highly localized concentration at particular sites prone to oxidants, whereas the binding to cation efficiently prevents the site-specific generation of ROS. Therefore, the enhanced level of PAs particularly Put, as observed in this study, perhaps help to maintain the membrane stability and permeability through binding to the negatively charged phospholipids head group. Groppa et al. (2007) also reported the increased level of endogenous Put in wheat leaves under cadmium and copper exposure.

One of the crucial factors of Cd^{2+} influence on plant metabolism and physiological processes is its relationship with other mineral nutrients. The uptake

and the intracellular concentration of essential metal species are kept under homeostasis to prevent the action of free ions as catalysis in Haber–Weiss and Fenton type reactions which otherwise results in oxidative injury in plants. In this study, the contents of minerals including Na, K, and Mn were significantly affected by Cd^{2+}. Foremost among these were the substantial increase of Na content and decrease of K. Considering their potential roles played in osmoregulation, variations in their content suggest that Cd influences the osmotic balance in the cell. The increased Mn content could positively be correlated with induced Mn-metalloprotein, i.e Mn-SOD isoforms and is in agreement with the findings of Apel and Hirt (2004). However, variation for Cd and Mn obtained in the present study requires further investigation, since Mn is known to involve in photolysis of H_2O by PSII or for the assimilation of NO_2^- in chloroplasts as well (Fodor 2002).

In conclusion, this study demonstrats that 0.4 mM Cd^{2+} concentration has induced ROS production and established some level of oxidative stress in *U. lactuca*. The tolerance against Cd^{2+} induced oxidative stress is due to increased activities of some of the major antioxidative enzymes like SOD, APX, GR, GPX and non enzymatic antioxidants which are involved in detoxification of ROS. However, strong inhibition of CAT following Cd^{2+} exposure invariably suggests the fine modulation of ROS for signaling by APX instead of CAT. Additionally, increased content of endogenous free and bound soluble Put following metal exposure suggest its role to keep the membrane stability and reduce the active oxygen generation more efficiently compared to that of Spm and Spd. Increased C18:3$n-6$ and C18:2$n-6$ fatty acids together with increased LOX activity with two new isoforms also provide evidence for LOX dependent lipoperoxide accumulation. Therefore, the results described in the present study together with the isoenzymes detected could be considered as possible biomarkers for monitoring the heavy metals in marine ecosystem.

Acknowledgments The financial support received from CSIR (NWP-018) is gratefully acknowledged. The first author (MK) and second author (PK) gratefully acknowledges the CSIR, New Delhi for awarding the Senior and Junior Research Fellowships respectively. The third author (VG) also expresses his gratitude to Department of Science and Technology, New Delhi for financial support.

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