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Antioxidant modulation in response to heavy metal induced oxidative stress in *Cladophora glomerata*

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The present investigation was carried out to study the induction of oxidative stress subjected to heavy metal environment. Lipoperoxides showed positive correlation at heavy metal accumulation sites indicating the tissue damage resulting from the reactive oxygen species and resulted in unbalance to cellular redox status. The high activities of ascorbate peroxidase and superoxide dismutase probably counter balance this oxidative stress. Glutathione and soluble phenols decreased, whereas dehydroascorbate content increased in the algae from polluted sites. The results suggested that alga responded to heavy metals effectively by antioxidant compounds and scavenging enzymes.

Keywords: Antioxidant, *Cladophora glomerata*, Heavy metal, Oxidative stress

Oxidative stress is characterized by increase in reactive oxygen species (ROS) as a result of insufficient antioxidant defense[1]. Responses of organisms to oxidative stress include the use of antioxidant enzymes, water-soluble antioxidant compounds and lipid-soluble antioxidant molecules. Antioxidant mechanisms in seaweeds have been poorly studied. Reports confirmed that desiccation causes oxidative stress in *Stictosiphona arbuscula*, *Mastocarpus stellatus* and *Chondrus crispus*[2,3]. Copper released from mines have reportedly caused severe effects in the coasts and at higher concentrations it becomes toxic[4]. Toxicity in algae is mediated through Haber-Weiss reaction, characterized by heavy metal-catalyzed production of hydroxyl radicals from hydrogen peroxide. Previous studies in *Ulva compressa* have demonstrated that the tolerance to copper is linked with antioxidant defenses to buffer the oxidative stress[5]. Such reports are missing for *Cladophora glomerata*. Therefore in this study, a biochemical mechanism for heavy metal tolerance in *C. glomerata* was examined.

Materials and Methods

Kalamukke in Cochin (9° 97′N 76°23′E), ashtamudi lake in Kollam (8° 53′ – 9° 52′N and 76° 31′ – 76° 41′ E) and munamakalle in Chetua, India were the sites selected for the study. Water and algal samples were collected in 250 ml acid washed polythene bottles and kept at 4°C during transportation. At the laboratory, the samples were immediately filtered using acid washed and pre-weighed millipore filters to separate particulate metals from the dissolved metals. Samples were analyzed for copper (Cu), iron (Fe), zinc (Zn), lead (Pb) manganese (Mn) and cadmium (Cd) by acid digestion and quantified using an atomic absorption spectrophotometer with Zeeman correction. The instrument was calibrated using analytical grade standards obtained from Merck. Recovery was tested using standard reference materials for estuarine water (SLEW 3 from NRC, Canada) and estuarine sediments (IAEA-405 from IAEA, Austria) and was found to be within the acceptable 90% confidence limits for all metals studied. Sample digestion for the determination of mercury (Hg) was done by cold atomic spectrophotometer in a mercury analyzer.

Estimation of lipoperoxides—Algal cells (1g dry weight) was frozen in liquid nitrogen and homogenized in a pre-chilled mortar using a pestle. Trichloroacetic acid [5 ml 0.1% (w/v)] was added during the homogenization. The homogenate was allowed to thaw at room temperature, transferred to a glass tube (30 ml) and centrifuged at 7400 *g* for 20 min. Lipoperoxides were detected by addition of 50-200 μl of the clear homogenate to a reaction mixture containing 0.5% thiobarbituric acid (solubilized in 20% trichloroacetic acid) in a final volume of 1 ml. The reaction mixture was incubated at 100ºC for 30 min and the absorbance

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was measured at 512 nm. To determine the amount of lipoperoxides, the extinction coefficient of the synthesized aduct was used (E=155 mM^{-1}cm^{-1}) [6].

Estimation of antioxidant compounds from algal tissue —GSH and GSSG levels were determined using dry tissue (0.5 g) as above except that 5 ml of 5% (w/v) sulpho-salycilic acid replaced TCA during homogenization. The homogenate was neutralized with 1.5 volumes of 500 mM phosphate buffer (pH 7). Total glutathione (GSH+ GSSG) was detected by addition of 50–300 μl of the homogenate to reaction mixture containing 100 mM, phosphate buffer (pH 7); 0.15 mM, NADPH; 60 μM DTNB; and 0.66 U, glutathione reductase (Sigma) to a final volume of 1 ml. The reaction mixture was incubated for 1 h at 37ºC and the absorbance was determined at 412 nm[5]. GSH was detected following the same procedure described above with the exception that 50–300 μl of neutralized homogenate were previously incubated with 20 μl of 2-vynil-pyridine (1 M) for 1 h at room temperature. The calibration curve was prepared using 2–40 nmole of GSH in the same reaction mixture.

Phenol compounds were detected using 0.1 g of fresh tissue. Algal samples were homogenized with a plastic pestle in an Eppendorf tube containing 1 ml phosphate buffer (0.1 M; pH 7). The homogenate was centrifuged in an Eppendorf microcentrifuge at 12800 g for 10 min. Aliquots of 50 μl were added to a reaction mixture containing 3% of sodium carbonate and 0.3 M Folin- Ciocalteau reagent in a final volume of 1 ml. The reaction mixture was incubated for 2 h at room temperature and the absorbance at 765 nm was determined. Total phenolic compounds were expressed as nanoequivalents of gallic acid using a calibration curve prepared with 10–50 nmole of gallic acid[7].

ASC and DHA were determined using 0.5 g of dry tissue. Algal samples were ground in liquid nitrogen and homogenized with 5 ml of 2.5 M perchloric acid. ASC was detected by adding 10-500 μl of the homogenate to a reaction mixture containing 2% (w/v), trichloroacetic acid; 8.8%, ortho-phosphoric acid; 0.01%, α,d- dipyridyl; and 10 mM, ferric chloride in a final volume of 1 ml. The reaction mixture was incubated for 1 h at 40ºC and the absorbance was determined at 525 nm. Total ascorbate (ASC and DHA) was detected following the same procedure described above except that 10–500 μl of the homogenate were previously incubated with 5 μl 100 mM dithiotreitol (DTT) for 1 h at room temperature. DTT was subsequently inactivated by addition of 5 μl of 5% (w/v) N-ethylmaleimide. The calibration curve was prepared using 10–300 nmole of ascorbate in the same reaction mixture[8].

Isolation and assay of antioxidant enzymes —Algal tissue (20 g fresh weight) was frozen in liquid nitrogen and homogenized in a pre-chilled mortar using a pestle. Phosphate buffer (60 ml of 100 mM; pH 7) containing 5 mM of 2-mercaptoethanol were added during the homogenization and filtered. Protein in the flow through fraction was precipitated by addition of ammonium sulphate /ml of extract. The protein pellet was solubilized in phosphate buffer (1 ml, 100 mM; pH 7) containing 2 mM of 2-mercaptoethanol and 10% glycerol. The final extract contained 2-4 mg /ml of protein. APX activity was determined by the addition of 2.5-25 μg protein extract to phosphate buffer (1 ml, 100 mM; pH 7) containing 0.4 mM, ASC; and 16 mM, H_2O_2 and decrease of absorbance at 290 nm due to ASC consumption was monitored for 1 min[9]. GR activity was determined by addition of 50-200 μg protein extract to 1 ml of 100 mM phosphate buffer (pH 7) containing 0.5 mM, GSSG; and 0.15 mM, NADPH and the decrease of absorbance at 340 nm due to NADPH consumption was monitored for 1 min[5]. CAT activity was determined by addition of 10–100 µg protein extract to 1 ml of 100 mM phosphate buffer (pH 7) containing 16 mM of H_2O_2 and the decrease in absorbance at 240 nm due to hydrogen peroxide consumption was monitored for 1 min[10]. DHAR activity was determined by addition of 10–100 μg protein extract to 1 ml of 100 mM phosphate buffer (pH 7) containing 1 mM, GSH; and 0.5 mM, DHA and the increase in absorbance at 290 nm due to ASC synthesis was monitored for 1 min. GPX activity was determined by addition of 10-100 μg protein extract to 1 ml of 100 mM phosphate buffer (pH 7) containing 0.1 mM GSH, 4.5 mM H_2O_2, 0.1 mM NADPH and 1U glutathione reductase (Sigma). The decrease in absorbance at 340 nm due to NADPH consumption was then monitored for 1 min[11]. Antioxidant enzyme activities were determined at 37º C using four replicates from each site. For each site, replicates consisted of extracts of composite *C.glomerata* samples collected the same day. Superoxide dismutase (SOD) was extracted and assayed following the method of Beyer and Fridovich[12].

Statistical analysis —The data were analysed using ANOVA.

Results and Discussion

The amount of heavy metals in the water bodies from three localities selected for this study clearly showed that almost all the metals tested remained as the major pollutants (Table 1). Levels of the metal concentration in *C. glomerata* from polluted and non-polluted sites are listed in Table 1. Bioaccumulation of minerals in the algae from polluted sites was at par with concentration of metals in water. i.e., non-polluted site showed comparatively lower levels. Thus, *C. glomerata* from polluted sites had average almost 2–3 times more metal ions than the control sites. Bioaccumulation of copper in algal tissue was positively correlated with its concentrations in water (r^2=0.7, p <0.05).

Concentration of lipid peroxides—Lipid peroxide of the alga from the polluted site was two fold higher (significant, F=60.6, p <0.00001) than non-polluted sites. There is a general agreement that a condition of oxidative stress results in the presence of abnormally high levels of oxidized macromolecules, including lipoperoxides[13]. Our results are consistent with this finding.

Activity of ROS—Scavenging enzyme activities of SOD, CAT, APX, GPX, DHAR and GR were determined in from the sites. CAT, GPX and DHAR had poor activities in *C. glomerata* irrespective of the tested sites of sampling. In the non-polluted site APX activity was low but it increased by over 10-fold in the polluted sites (Table 2). The GR activity showed an opposite trend (Table 2). Inhibition of GR activity, which probably results from a direct action of heavy metals on the enzyme, as it has been documented for GR in *Euglena gracilis* and in some terrestrial algae[14]. SOD showed a steady increase in the activity from non-polluted to polluted site suggesting the higher scavenging activity of $O_2^{\cdot-}$ to H_2O_2 (Table 2). SOD can indicate the extent of pollution which may induce $O_2^{\cdot-}$levels[15]. An increase in the activity of antioxidant enzymes (SOD and APX) might reduce the concentration of $O_2^{\cdot-}$ and H_2O_2, thereby minimizing the risk of OH. through cycling between transition metals.

Estimation of total phenol content and glutathione—Total glutathione pool and levels of reduced (GSH) and oxidized glutathione (GSSH) were significantly higher in non-polluted sites indicating heavy metals carry an impact on water soluble antioxidant compounds (Table 2). The concentration of glutathione in samples from polluted site was highly diminished compared to those collected from non-polluted site. Similarly, the mean total phenol in

Table 1—Heavy metals in water sample and algal tissue from the study sites. Each value in ppm of dissolved metals represents the concentration measured in a composite sample resulting from combining three samples collected from different sites

	Surface water(mg/l)			Algal tissue (µg/g)		
	Cochin	Kollam	Chetua	Cochin	Kollam	Chetua
Copper	86.3	71.4	35.6	53.6	51.5	14.1
Cadmium	18	18.4	10.9	4	3.8	1.4
Lead	63.7	79.3	37	4.6	5.1	1.7
Mercury	0.2	0.17	0.03	0.07	0.08	0.004
Zinc	337.4	292.9	242	0.7	1.1	0.89
Iron	46.6	48	17.3	40.4	4.4	12.3
Manganese	408.7	430.7	215	1.4	3.1	0.45

Table 2—Effect of heavy metals on antioxidant enzymes and compounds in *C.glomerata* from polluted and control sites (Values are mean ±SE from six samples collected from different Sites)

Parameters	Kollam	Cochin	Chetua
APX (moles ascorbate $min^{-1}mg^{-1}$ protein)	56.6±1.07	38.6±0.89	1.8±0.2
GR (µ moles NADPH $min^{-1}mg^{-1}$ protein)	0.2±0.02	0.28±0.03	0.57±0.01
SOD (units/mg protein)	15.5±0.51	15.1±0.02	2.1±0.01
LPX (nmoles g^{-1} DW)	289.4±1.28	308 ±1.3	153.7±1.05
GSH (µ moles g^{-1} DW)	0.011 ±0.001	0.013±0.001	2.85±0.02
GSSH (µ moles g^{-1} DW)	0.012±0.001	0.1 ±0.001	2.63±0.001
Phenol (µ eq of GA g^{-1}FW)	0.85 ±0.01	0.7±0.01	5.6±0.01
ASC (µ moles g^{-1} DW)	2.1 ±0.02	1.9 ±0.01	4.5±0.01
DHA (µ moles g^{-1} DW)	143.5 ±0.69	147.8 ±1.21	4.9±0.01

(*P* values: < 0.00001 F ratio: APX-186.2; GR-89.4; SOD-103.7; LPX-60.6; Total Glutathione-16453; phenol-2468; Total ASC-614)

non-polluted site was 10-fold higher than the polluted sites (Table 2). The total ascorbate pool (ascorbate+dehydroascorbate) in the polluted site showed a higher level of dehydroascorbate (DHA) than ascorbate. The amount of DHA in material from the polluted site, increased to a very high level (20 - fold; Table 2). Therefore, it was suggested that in this species oxidative stress involved synthesis of ASC that accumulated as DHA[5]. An important aspect that adds relevance to some of the changes observed in the antioxidant compounds is the potential relationship between glutathione and phytochelatins. As already suggested by Ratkevicius et al[5] working with *Enteromorpha compressa*, the significant consumption of GSH and GSSG could be explained by the channeling of GSH to phytochelatins, which are glutathione polymers known by their metal chelating properties and by their ROS scavenging activity, which is reportedly higher than GSH. Another possibility is that GSH synthesis is inhibited, although so far no evidence for a direct inhibition by copper of the enzymes involved in GSH synthesis has been reported in algae.

The combined activation of SOD and APX is expected based on the tight coupling between $O_2^{\cdot-}$, H_2O_2 and ascorbate. The enhanced APX from polluted sites with increased DHA content strongly suggested the oxidation of ascorbate by APX. The regeneration of ascorbate from DHAR activity coupled to APX activity is probably not occurring in alga thriving in polluted sites due to the apparent absence of DHAR. The accumulation of high levels of DHA may be due to direct oxidation of ascorbate by ROS[16].

In conclusion, the macro green alga *C.glomerata* constantly exposed to heavy metals tolerates metal induced ROS stress by quenching oxygen radicals by SOD and APX by synthesizing ascorbate, consuming GSH and accumulating heavy metals.

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