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THE INFLUENCE OF METALS ON THE ANTIOXIDANT ENZYME, SUPEROXIDE DISMUTASE, PRESENT IN THE CYANOBACTERIUM, *Anabaena variabilis* KÜTZ

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ABSTRACT

Cyanobacteria have a highly evolved antioxidant system that catalyzes the harmful oxy radicals produced during photosynthesis. The differential response of superoxide dismutase (SOD), nitrogenase, growth and physiological processes in the presence of four different metals ions at varying concentrations were investigated in the heterocystous cyanobacterium, *Anabaena variabilis* Kütz. Growth and enzyme activities were influenced by the metal ions. SOD activity was optimum at 100 mcM concentration of $FeSO_4$ but nitrogenase activity decreased in the presence of the metal ions. SOD activities increased with increasing concentrations of $MnSO_4$ while nitrogenase activities decreased when compared with control. In the presence of $CuSO_4$, all the enzyme activities were increased compared with control and optimal enzyme activity was observed at 300 mcM concentration. In the presence of $ZnSO_4$, nitrogenase and glutamine synthetase activities reduced with increasing concentrations of the metal salt while SOD activity increased in direct proportion with metal concentration. The differential expression of FeSOD and MnSOD in the cells of *A. variabilis* were also investigated by electrophoresis and are presented in this paper.

Keywords: *Anabaena variabilis*, cyanobacteria, metals, nitrogen-fixing enzymes, superoxide dismutase.

INTRODUCTION

Cyanobacteria are oxygen evolving photosynthetic prokaryotes and produce reactive oxygen species (ROS) that can damage cellular components leading to cell death. Therefore, the co-evolution of an antioxidant system occurred with the ubiquitous metalloenzymes, SODs, catalyzing the disproportion of superoxide radicals to peroxide and molecular oxygen through alternate oxidation and reduction of their metal ions. Cyanobacteria possess an effective stress combat system to cope with this pressure by the help of a cascade of antioxidants, where the SODs act initially followed by the catalase and peroxidases. In 1969, Mc Cord and Fridovich discovered the catalytic activity of SOD and named it as such. SOD is actively involved in the defense of the organism against oxy radicals (O_2^-). SOD was first commissioned in 1985 to be researched as an enzyme drug in the USA for defence of donor organs against oxidative stress during periods of ischemia and reperfusion (Vellard, 2003). SODs are highly valued as therapeutic 'enzyme drugs' (Bannister et al., 1987; Proctor and Reynolds, 1984). O_2^- radicals are unable to cross the cytoplasmic membranes and it becomes imperative that they are removed from the source and this may have contributed to the compartmentalization of SOD enzymes (Takahashi and Asada, 1983; Hassan and Fridovich, 1979). Thus, a clear demarcation of the territorial boundaries of the SOD isozymes seems to have been chalked out. ROS formed in the biological system during the normal course of metabolism are potentially harmful since they attack membranes, proteins and DNA molecules. ROS formation is also increased in the presence of trace amounts of iron or other transition metal ions and metal chelators (Padmapriya and Anand, 2009). Metal salts have been known to induce the SOD enzyme in bacterial cells. Increased intracellular concentrations of oxy radicals might be the reason for the triggering of the enzyme in the cells. In the present study, the response of the growth, antioxidant and the nitrogen-fixing enzymes of *Anabaena variabilis* grown in the presence of the sulphate salts of copper, zinc, iron and manganese were investigated.

MATERIALS AND METHODS

The *A. variabilis* cultures were obtained from the Culture Collection at the Center for Advanced Studies in Botany, University of Madras, Chennai, India. All chemicals and reagents were obtained from Sisco Research Laboratories, Mumbai, India. Cultures were grown and maintained in nitrate-free BG_{11} medium (Rippka *et al*., 1979). Cultures were maintained at 27°C±1°C under fluorescent illumination of 30-40 $\mu Em^{-2}s^{-1}$ provided by fluorescent tubes (Philips Trulite, Col 82), exposed to a 12 h light / 12 h dark photoperiod and swirled manually for five minutes, thrice daily. Growth was determined every fourth day up to 20 days by measuring the chlorophyll a content (Mc Kinney, 1941). Protein estimation was carried out using bovine serum albumin as standard and the results were expressed in µg/mL (Bradford, 1976). Photosynthetic oxygen evolution and respiratory oxygen consumption in the intact filaments were measured at 27ºC using Clark type Oxygen Electrode (YSI model 53) at a light intensity of $100\mu Em^{-2}s^{-1}$ (Allen and Holmes, 1986). Hydrogen peroxide estimation was carried out in the extracellular medium and the extinction coefficient of the quinoneimine dye (product) at 505nm was taken as $6.4x\ 10^3 M^{-1}cm^{-1}$ (Frew *et al*., 1983). Nitrogenase activity was measured in whole cells by the Acetylene Reduction Assay (ARA) (Stewart *et al*., 1967) and the activity was expressed as nM of $C_2H_4\ h^{-1}$ mg Chl a^{-1}. Glutamine synthetase (GS) was measured by the

biosynthetic assay and 1U of the enzyme corresponded to 1µg Pi formed min^{-1} (Shapiro and Stadtman, 1970). The activity was expressed as U mg $protein^{-1}$. The SOD activity was expressed in units (U) where 1U corresponded to the amount causing half the maximum inhibition of nitroblue tetrazolium (NBT) to blue formazan. The activity was calculated using the formula- U/mL = [(V_0/v) - 1] (dilution factor); V_0: A_{560} of control, v: $A_{560\ of}$ sample. The results were expressed as U mg $protein^{-1}$ (Beauchamp and Fridovich, 1971). The polyacrylamide gel electrophoresis (PAGE) for native gel-assay was carried out under a constant current of 10 mA under non-denaturing conditions. SOD bands appear colorless against a blue background and isozymes were detected by the method of Beyer and Fridovich (1987). The gels were cut and soaked in 2 mM of KCN or 2 mM of H_2O_2 for 10 mins prior to soaking in NBT. FeSOD is inactivated by H_2O_2; Cu-ZnSOD is inactivated by KCN while MnSOD remains unaffected by these compounds. All the experiments were conducted in triplicates. Standard deviation and One-Way ANOVA were used for the statistical analysis of the data.

RESULTS

Groth of *A. variabilis* at 100µM concentration of $FeSO_4$ was significantly decreased compared with control. There was a approximately 22% decrease in chlorophyll *a* content at $FeSO_4$ 100µM treated cells when compared to control (Table-1). Protein content was also reduced at $FeSO_4$ 100µM treated cells. Reduced photosynthesis rates were observed in all the $FeSO_4$ treated cultures. Respiratory rates, though reduced in treated cells, were not significantly decreased. Peroxide concentrations in the external medium were significantly decreased by 2.5-2.3 folds in treated cells. Both SOD and GS showed an increase in their activities at $FeSO_4$ 100µM concentration (Table-2). Nearly 2.5 folds increase in SOD activity and 1.4 folds increase in GS activity were observed at $FeSO_4$ 100 µM compared with control. At $FeSO_4$ 500µM treatment, a significant decrease in enzyme activities was observed where the activities of GS decreased by 65%, nitrogenase by 70% and SOD by 45% (Table-2). The detection of the isozymes of the antioxidant enzyme is shown in Fig 1. Both the MnSOD and FeSOD bands were clearly visible in the native PAGE assays. The intensity of the bands of FeSOD was higher in the control cells which decreased as the metal concentration increased. Two distinct bands of FeSOD were visible in all the lanes.

$MnSO_4$ decreased protein content by ~10% and chlorophyll *a* content by ~17%-24% compared to control (Table-1). There was significant decrease in photosynthetic rates by ~47% at $MnSO_4$ 500µM treatment compared with control. Increased respiratory rates were observed in all the $MnSO_4$ treatments. Peroxide levels were significantly increased by ~72% at $MnSO_4$ 100µM treatment compared to control (Table-1). A linear increase in the SOD enzyme activity with increasing $MnSO_4$ concentrations was observed. A ~12-fold increase of the SOD enzyme activity was seen at $MnSO_4$ 500µM when compared with control ($p<0.01$) (Table-2). Significant decrease of GS activity by ~75% at $MnSO_4$ 100µM treatment compared with control was observed. Nitrogenase activity was also decreased in $MnSO_4$ treated cultures when compared to control (by ~20% at $MnSO_4$ 500µM treatment). Native PAGE assays for the SOD isozymes are shown in Figure-2. There were 2 distinct bands of FeSODs detected in all the lanes. The MnSOD bands were clearly visible in the control and 100 uM-treated culture lanes but at higher concentrations (lanes 3 and 4), there was distinct smudging of highly colorless zones at the site of MnSOD bands probably indicating a very high enzyme activity in these high metal concentration cells.

In experiments with copper ions, it was observed that protein and chlorophyll *a* content were significantly decreased in $CuSO_4$-treated cells when compared to control. At $CuSO_4$500µM concentration, protein content decreased by ~9 folds and chlorophyll *a* decreased by ~3 folds when compared to control (Table-1). Photosynthesis was completely inhibited in $CuSO_4$-treated cultures. Presence of peroxide in the medium was observed to increase in a linear fashion with increasing $CuSO_4$ concentrations where the highest increase by 133 folds was observed at 500µM concentration. Significant decreases in respiratory activities were also observed at 500µM treated cells (~54%) (Table-1). Enzyme activities were found to be significantly high in $CuSO_4$-treated cells. At $CuSO_4$ 300µM treatment, GS activity increased by ~5 times compared with control. SOD activity increased by ~116 folds in $CuSO_4$ 300µM treated cells and nitrogenase activity increased by ~4.5 folds in $CuSO_4$ 500µM treatment. Figure-3 shows the SOD isozyme activity in the gel assays. The FeSOD and MnSOD activities and band intensities were distinctly reduced in the treated cells compared with control. The band width of MnSOD in the 300µM and 500µM treated cells was more diffuse compared with the bands in the other 2 lanes. 2 distinct FeSOD bands were observed in the control lane only but not in the other lanes (Figure-3).

Zinc sulphate treatment was detrimental to the growth of *A. variabilis*. Protein and chlorophyll *a* levels were significantly decreased by ~9 folds and by ~1.72 folds, respectively, at $ZnSO_4$ 500µM treatment compared with control (Table-1). Total absence of photosynthesis was observed in the treated cultures. Increased respiratory rates at $ZnSO_4$ 500µM-treated cells were observed (~40%) along with a significant increase in peroxide levels (~2.8 folds) compared with control. GS activity was significantly low in the presence of $ZnSO_4$ metal salts while SOD activities increased. Nitrogenase activity was observed to be significantly decreased in the $ZnSO_4$-treated cells reaching low levels of ~2.1nM C_2H_4/h/ µg chl.*a* at 100µM treatment (Table-2). In-gel assay of the soluble extracts of treated cells showed the prominent presence of both the FeSOD and MnSOD enzymes. Clear, distinct bands of MnSOD were observed in the control and 100 µM-treated cells (lanes 1-2) but smudges of colorless zones at the site of MnSOD bands were observed in lanes

3 and 4, probably indicating high enzyme activity. The FeSOD bands were similar in intensity in all the lanes and 2 distinct bands were detected at the FeSOD zone (Figure-4).

DISCUSSIONS

Trace metals play an important role in the regulation of elements in the various metabolic processes and affect the uptake of macronutrients (Mc Kay *et al*., 2001). Availability of micronutrients *viz*. Mn, Fe, Co, Ni, Cu and Zn determine the productivity based on macronutrient (Bruland *et al*., 1991; Sunda, 1994, 2000). Besides these nutrients, cyanobacteria are known to require molybdenum, boron and vanadium. However, iron, copper, zinc and manganese have been regarded as essential elements since earlier times (Eyster, 1964; O'Kelly, 1974). SODs may act as markers of functional metals along with their role as effective models to study the mineral nutritional requirements and physiology of the organism (Almansa). Of the four trace metals used in the present study it was observed that copper had the maximum inhibitory effect on growth (determined as chlorophyll and protein content) of *A. variabilis*. Iron seemed to stimulate growth in the organism. Mo followed by Fe, Mn, Co and Zn stimulated primary productivity in lakes (Goldman, 1965). However, peroxide content was the highest in copper and zinc-amended cultures. Fe and Mn amended cultures showed less amount of peroxide content in the cultures. Photosynthesis was completely inhibited in copper and zinc amended cultures but a continuous low level of photosynthesis was observed in Fe and Mn cultures. There was a decrease in chlorophyll *a* and protein contents of *Synechococcus* cells grown in the presence of elevated levels of zinc (Chintamani and Mohanty, 1988). The response of *A. variabilis* to the micronutrients was varied with reference to the activities of nitrogenase, GS and SOD. An increase in SOD activity under all the amendments was noticed. Copper induced the maximum SOD, GS and nitrogenase activity while induction by iron was comparatively low. Studies on *Chlorella vulgaris* showed a concentration dependent increase in SOD activities when exposed to copper (Nirupama Mallick, 2004). GS activity was affected by high concentrations of iron, zinc and manganese to an extent while nitrogenase activity was reduced in the presence of Fe, Mn and Zn. In a study on the effect of copper on the antioxidant system in *Anabaena doliolum*, there was nearly 63% increase in SOD activity at 1.0mg/ml concentration of copper (Mallick and Rai, 1999). Similar increase in SOD activity was observed in the marine dinoflagellate *Gonyaulax polyedra* (Okamoto and Colepicolo, 1998). A rise in these antioxidant enzyme levels suggests that there is an excess of toxic oxygen species that has to be scavenged (Halliwell and Gutteridge, 1999). Though there is Cu-induced activation of SOD, catalase and carotenoid synthesis, significant oxidative damage occurs in the cell with increase in lipid peroxidation (Mallick and Rai, 1999). In the present investigations similar results were observed where Cu and Zn at high concentrations resulted in bleaching of pigments and finally degradation of cells. The isoforms of the SOD enzyme in the marine cyanobacterium-*Synechococcus* sp., in the absence of iron in the growth medium did not show any alteration in the FeSOD activity but absence of Cu or Zn in the growth medium resulted in differential changes in the activities of Cu/Zn SOD and the unidentified SOD (Chadd *et al*., 1996). In another study, the transcript levels of the FeSOD (*sodB*) were decreased under iron limiting conditions suggesting a down regulation of the gene expression in response to lack of available iron within the cell (Campbell and Laudenbach, 1995). The cells of *A. variabilis* showed only faint FeSOD bands when grown under Cu amendments. This is because a high degradation of the cells resulted due to toxicity though the specific activity of the enzyme was very high. The presence of 2 kinds of SOD in cyanobacteria appears to be an advantage for adapting effectively to the fluctuation of metal concentration in the environment (Li *et al*., 2002). In conclusion, the present study reveals that the metal ions in the immediate environment play an important role in influencing the activity of the SOD enzyme. The four metal salts had a differential effect on the growth, antioxidant and nitrogen-fixing enzymes in *A. variabilis*.

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Table - 1. Growth [Chlorophyll *a* and protein- µg/mL], oxygen rates [photosynthesis and respiration- nM O_2 ↑↓/h/mg Chl *a*] and extracellular peroxide [µM/mL] levels of the cyanobacterium *A. variabilis* grown under various concentrations of metal salts. Values are mean ± standard deviation. All the results were statistically significant at $p<0.05$ using ANOVA: single factor.

Metals	**Conc** (mcM)	**Chl a**	**Protein**	**PS**	**RS**	**Peroxide**
$FeSO_4$	0	1.25±0.03	13.26±1.6	185.0±41.0	66.0±3.1	0.97±0.2
	100	0.97±0.2	12.31±1.7	86.13±18.0	55.53±8.75	0.4±0.01
	300	1.57±0.28	20.31±0.5	143.92±4.0	54.89±10	0.3±0.2
	500	1.54±0.11	13.55±1.9	144.2±10.0	44.59±3.9	0.46±0.3
$MnSO_4$	0	1.14 ±0.1	26.15±2.5	273.64±89.1	64.56±14.9	1.5±1.0
	100	1.18±0.03	23.71±2.9	119.85±3.4	114.23±76.7	2.5±0.6
	300	0.86±0.06	23.46±3.4	114.21±44.6	81.75±32.7	1.7±0.3
	500	0.97±0.1	24.12±1.9	128.26±37.9	65.58±23.4	0.75±0.3
$CuSO_4$	0	4.27±0.06	46±1.9	74.8±8.1	40.1±18.4	0.17±0.01
	100	3.12±0.3	10.6±0.1	-	28.4±16.0	6.53±3.1
	300	2.32±0.01	8.1±2.5	-	32.7±15.1	20.7±1.7
	500	1.52±0.03	5.41±0.9	-	18.3±8.1	22.6±1.1
$ZnSO_4$	0	1.1±0.4	46±17.5	287.37±20.6	121.5±22.5	11.5±1.1
	100	1.61±0.04	20.86±9.1	-	79.85±36.5	10.4±1.5
	300	1.16±0.3	6.15±2.1	-	89.5±32.2	7.8±2.1
	500	0.57±0.01	5.15±3.5	-	174.0±82.3	28.8±3.3

Table-2. Activities of the enzymes - glutamine synthetase [(GS)-U/µg protein], nitrogenase [nM C_2H_4/h/µg Chl. *a*] and Superoxide dismutase [(SOD)-U/mg protein] in the cyanobacterium, *A. variabilis* grown in varying concentrations of metal salts. Values are mean ± standard deviation. All the results were statistically significant at $p<0.05$ using ANOVA: Single factor.

Metal salt	**Concentration**	**GS**	**Nitrogenase**	**SOD**
$FeSO_4$	0 mcM	0.4±0.03	3.17±0.3	4.07±0.15
	100µM	0.56±0.04	0.44±0.03	10.25±0.3
	300µM	0.05±0.02	0.39±0.02	6.75±0.25
	500µM	0.13±0.01	0.95±0.02	2.26±0.16
$MnSO_4$	0 mcM	0.41±0.03	9.4±0.17	4.07±0.6
	100µM	0.11±0.01	7.8±0.14	18.5±1.7
	300µM	0.10±0.01	9.1±0.43	49.2±1.3
	500µM	0.58±0.02	7.6±0.21	72.94±3.3
$CuSO_4$	0 mcM	0.79±0.03	2.31±0.4	4.88±0.7
	100µM	1.1±0.2	5.12±1.0	171.18±1.5
	300µM	3.88±0.08	5.66±1.4	567.45±2.5
	500µM	2.38±0.3	8.88±1.9	361.54±16.5
$ZnSO_4$	0 mcM	0.79±0.03	10.25±0.7	4.88±0.7
	100µM	0.56±0.02	2.16±0.3	87.72±1.6
	300µM	0.11±0.03	4.4±0.3	57.0±3.4
	500µM	0.17±0.02	6.5±1.0	19.0±1.7

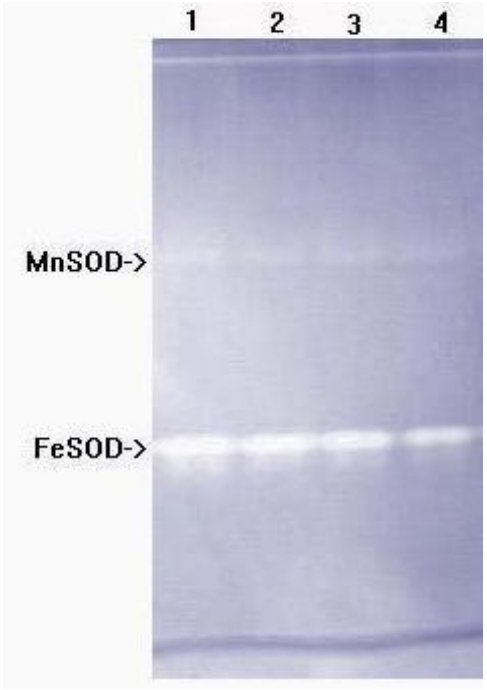

Figure-1: Native page showing the isozymes of SOD in *A. variabilis* treated with ferrous sulphate.

Lane 1: Control, Lane 2: 100µM,
Lane 3: 300µM, Lane 4: 500µM

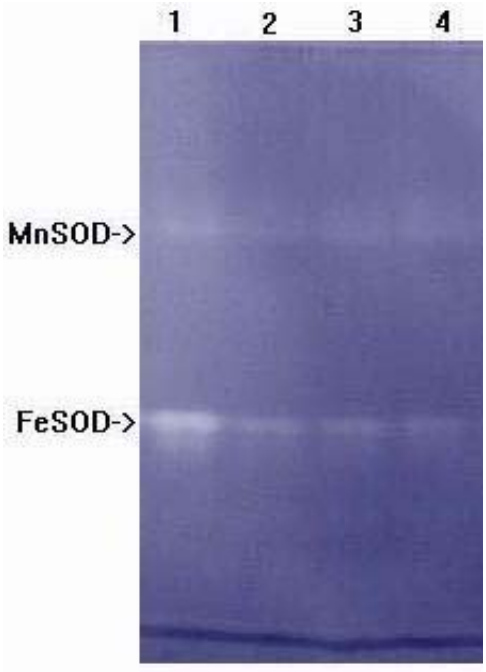

Figure-3. Native page showing the isozymes of SOD in *A. variabilis* treated with copper sulphate.

Lane 1: Control, Lane 2: 100µM,
Lane 3: 300µM, Lane 4: 500µM

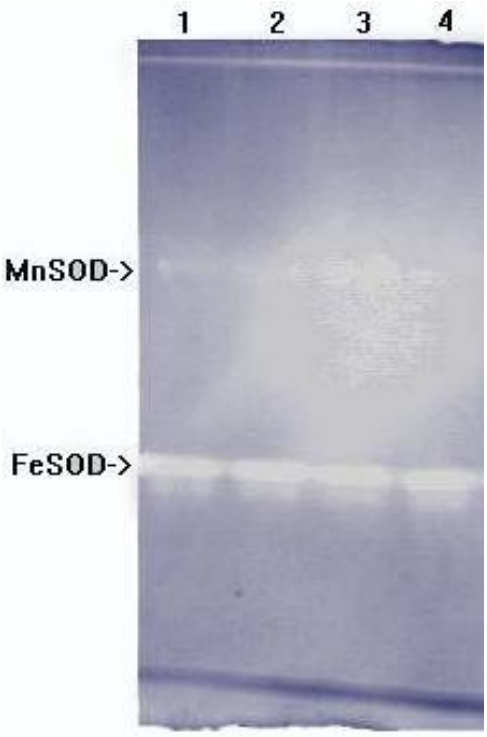

Figure-2. Native page showing the isozymes of SOD in *A. variabilis* treated with manganese sulphate.

Lane 1: Control, Lane 2: 100µM,
Lane 3: 300µM, Lane 4: 500µM

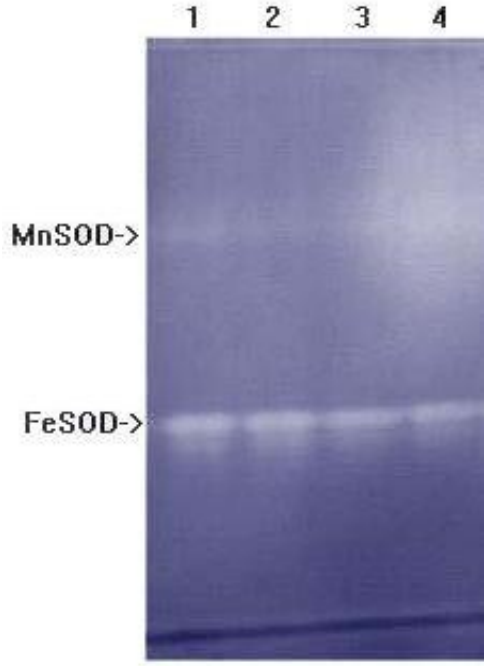

Fig 4: Native PAGE showing the isozymes of SOD in *A. variabilis* treated with Zinc sulphate.

Lane 1: Control, Lane 2: 100µM,
Lane 3: 300µM, Lane 4: 500µM.