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Time Course Biochemical Responses of Green Algae *Scenedesmus obliquus* to Aluminum and Low pH

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Acidic precipitation caused by atmospheric pollution increases the availability of metals in aquatic ecosystems. The increase in soluble Al in waters of low pH induced by acidic precipitation is known to be highly toxic and detrimental to aquatic communities (Jones and Kochian 1997). Aluminum is one of the most important growth-limiting factors of plant and algae in acidic water (Dong et al. 2002). Recent studies have focused on the physiological mechanisms of aluminum toxicity (Richards et al. 1998). Many authors have suggested that acidic precipitation may induce the response of antioxidant system in cells for clearing up excessive free radicals in plants caused by high concentration of aluminum and low pH.

Recently, the development and use of biomarkers has become a major interest in assessing the risks of exposure to environmental stress. The initial effects of the environmental stress on an organism are displayed normally as changes at the biochemical level of cell function prior to the appearance of visible morphological alterations. Free radicals are the result of normal oxygen metabolism in aerobionts. Correspondingly, aerobionts develop antioxidant defenses to maintain a homeostasis of redox status to protect itself from oxidation harm (Winston et al. 1991). Many documents have shown that impairment of stresses such as acidic precipitation, on some organisms is through the accumulation of free radicals. Superoxide dismutase (SOD) and peroxidase (POD) are the enzymatic constituents and glutathione (GSH) is a non-enzymatic constituent of the antioxidant system and responsible for scavenging free radicals. SOD firstly catalyzes the fundamental free radical to H_2O_2 and then POD and GSH sequentially convert H_2O_2 to harmless O_2 and H_2O using electron donors. These constituents are induced by the existence of free radicals and their level in cells represents a promising bioindicator to environmental stress.

It is reasonable to hypothesize that the oxidative stress induced by high concentration of aluminum and low pH occurs gradually with the time of exposure. Monitoring the biochemical response, such as the activity of these enzymes, may not only serve as the basis for environmental impact assessment but may also provide early warning signals before morbidity or mortality is visible in a population. It is possible that the effects of pollution on cellular metabolism

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may therefore be considered to be a reliable biomarker in monitoring the level of acidic precipitation in the environment (Kong et al. 1999).

The objective of this study is to investigate the time course biochemical response of the green alga *Scenedesmus obliquus* to the combined effects of Al and low pH. After exposure to various Al concentrations at different pHs, the protein content and activities of SOD, POD and the content of GSH were measured. These enzymes and constituents are associated with the antioxidant defense systems in cells, thus a possible sequential effect of Al and low pH on algae is discussed.

MATERIALS AND METHODS

Scenedesmus obliquus kutz was obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, cultured in 80 mL liquid HB-4 medium into 250 mL flasks kept on a rotator shaker at 25 ℃ and illuminated with cool-white fluorescent lights (75 μ mol photon m^{-2} s^{-1}) at 14:10 LD cycle. The culture medium was sterilized at 121℃, 1.05 kg cm^{-2} for 30 min. The culture medium consisted of 200 mg L^{-1} $(NH_4)_2SO_4$, 30 mg L^{-1} $Ca(H_2PO_4)_2$, 80 mg L^{-1} $MgSO_4$, 100 mg L^{-1} $NaHCO_3$ and 25 mg L^{-1} KCl (Kong et al. 1999).

The pH treatments were done by adjusting the pH of the medium with 1 M HCl to pH 6.5, 5.5 and 4.5 respectively before and after autoclaving. Al as $[Al_2(SO_4)_3]$ and HCl was introduced into the medium to get the expected concentrations of Al^{3+} (0.0, 0.25, 0.7, 1.4 mg L^{-1}) and pH values. The flasks were inoculated with 1 mL of fresh algal cells. The beginning density of algal cells was $3.0\text{-}4.0\times10^6$ L^{-1} and the flask was incubated on a shaker (Kong et al. 1999). The algal cells were collected by centrifuging at different time of exposure 0, 24, 48, 72 and 96 hr for assay.

Protein content in the algal cells was determined by the method of Lowry et al. (1951) and with bovine serum albumin (BSA) as standard. Protein content was expressed in μ g per mg dry weight of algal cells.

For enzyme assays, the algal cells were collected and extracted with 1mL of Tris/ Borate (0.1 M/0.3 M, pH 7.5, 5 mM EDTA, and 7 mM beta-mercaptoethanol) buffered on ice for 10 min. The extracts were then centrifuged at 10,000 g for 10 min at 4 ℃ and the clear supernatant were stored at –58 ℃ for the assay.

Superoxide dismutase (SOD, EC 1.15.1.1) was assayed with an improved method of pyrogallol autoxidation as described by Zou et al. (1986). Peroxidase (POD, EC 1.11.1.7) was assayed as described by Silberstein et al. (1996). One mL of reaction mixture contained 40 mM phosphate buffer (pH 7.0), 4 mM H_2O_2, 4 mM pyrogallol and 0.2 mL enzyme extract. The reaction was started by adding pyrogallol, and the optical density change in unit time was measured at 470 nm. Activities of enzyme were expressed in U $min^{-1}mg^{-1}$.

Glutathione (GSH) was assayed by a modification of the method of Zhang et al. (1994). The fluorescence of the solution was measured immediately in a spectrofluorometer (SHIMADZU RF-500) at 343 nm (excitation) and 425 nm (emission) after 1 min incubation. A standard curve was drawn to determine the GSH content. All biochemical compounds used in the experiment were from Sigma (USA).

Analyses were carried out in triplicate for each sample and the experiment was repeated twice. Statistical analysis was carried out using a one-way ANOVA with the SPSS software. The data were re-analyzed by a two-way ANOVA executed for the interaction among Al concentration, pH and treatment-by-time course for SOD, POD, GSH and protein. Data were expressed as means ± S.E bars. Probability values of less than 5% were considered significant.

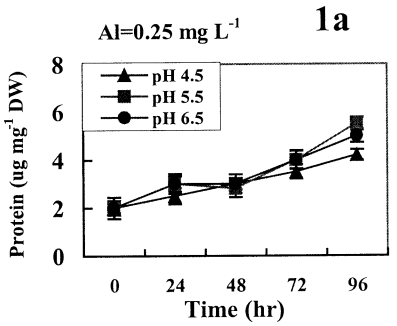
RESULTS AND DISCUSSION

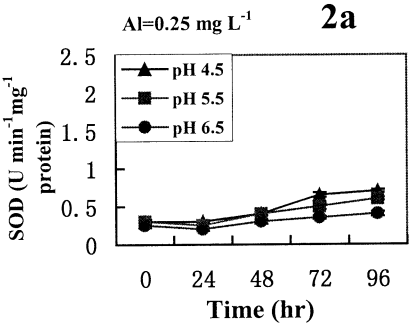
The response of the protein content to the Al concentrations, pH and exposure time are presented in Fig.1. Protein content decreased with Al concentration and acidity in the order of pH 4.5 < 5.5 < 6.5. It is very interesting to note that an increase in protein content with the treatment of 0.25 mg L^{-1}Al concentration was found. The treatment of 0.7 mg L^{-1} Al initially increased the soluble protein concentration for the first 48 hrs at the pHs tested, after that, the protein decreased with time (Fig.1b). However, increasing the Al concentration to 1.4 mg L^{-1} dramatically reduced protein content in the algal cells during the time of exposure ($p < 0.001$).

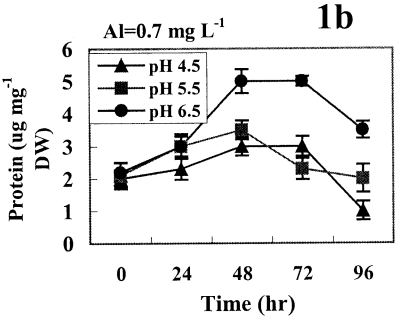
Reduction in protein content was found to be more pronounced at pH 5.5 (Fig. 1c, $p<0.001$). Reports on the effects of Al on protein content in plants are contradictory. Assche et al. (1988) reported that protein concentrations increased in roots and leaves after the application of metals such as Cu, Al and Zn. Taylor et al. (1997), however, reported decreased protein synthesis over a broad range of stress conditions. The findings here are in agreement with Kong et al. (1999) who showed that at Al concentration of 0.7 mg L^{-1} protein content in *S. obliquus* was higher than in the control.

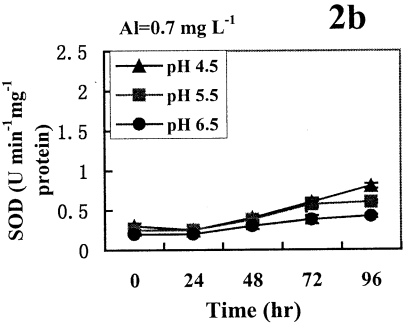
The activities of SOD, POD and GSH were stimulated significantly after treatment with Al and pH during all the times of exposure (Fig. 2, 3, and 4). SOD activity increased with Al concentration and acidity in order of pH 4.5 > 5.5 > 6.5. The enzyme activity was higher at 1.4 mg L^{-1} of Al than at 0.25 mg L^{-1} for all the pHs tested. Here, SOD activity was 2.12 times higher at pH 4.5, 1.84 times higher at pH 5.5 and 1.66 times higher at pH 6.5 respectively ($p < 0.001$). SOD activity also increased with time of exposure of the algae from 0-96 hrs at high Al concentration.

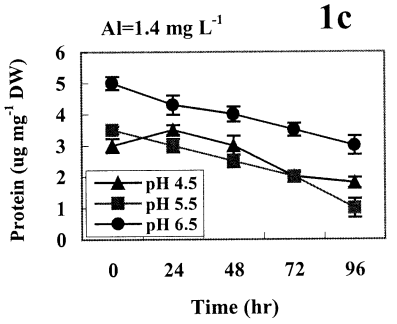
POD activity similarly increased at high Al concentration with the time of exposure (Fig. 3c). There was a very good correlation between POD activity and time of Al and pH exposure. POD activity was also found to increase with











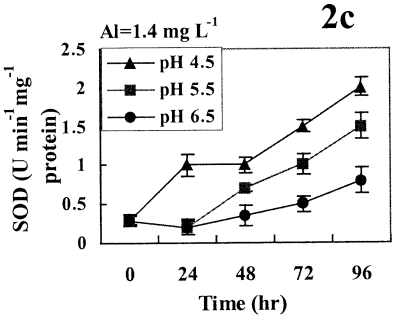
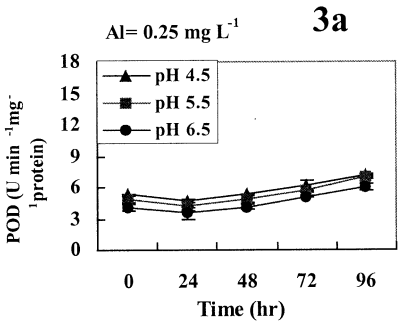


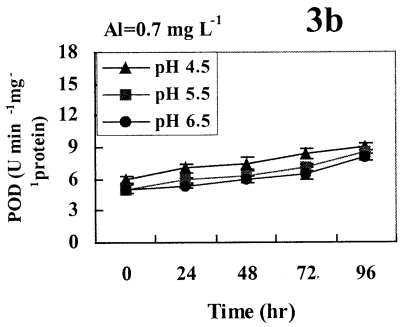
Figure 1. Time course of protein content in *S. obliquus* at different Al concentration and pH. Values are means ± S.E (n=15).

Figure 2. Time course of SOD activity in *S. obliquus* at different Al concentration and pH. Values are means ± S.E (n=15).

increase in Al concentration and acidity in the order pH 4.5 > 5.5 > 6.5. The highest activity was observed at pH 4.5 and 1.4 mg L^{-1} Al and this was found to be significant.

GSH content similarly showed elevated levels in order of pH 4.5 > 5.5 > 6.5 (Fig. 4). However when the Al concentration was increased to 1.4 mg L^{-1} (Fig. 4c), the GSH content was lower than the treatment with 0.7 mg L^{-1} Al at pH 5.5 and 6.5





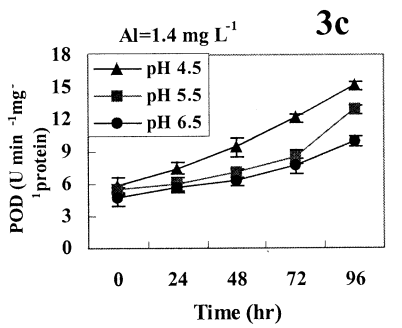
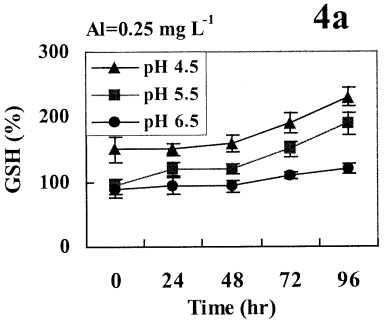
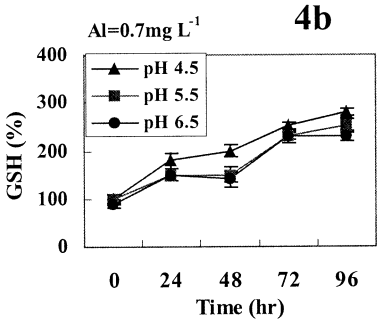


Figure 3. Time course of POD activity in *S. obliquus* at different Al concentration and pH. Values are means ± S.E (n=15)





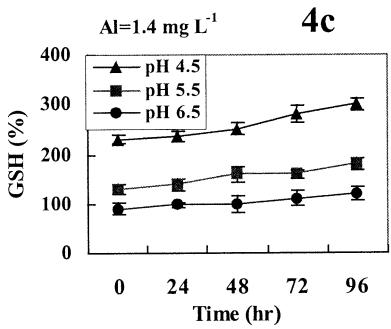


Figure 4. Time course of GSH content in *S. obliquus* at different Al concentration and pH. Values are means ± S.E (n=15)

after 96 hrs (Fig 4b and c, $p < 0.001$). GSH content at 0.7 was twice as that at 1.4 mg L^{-1} at pH 5.5 and 6.5.

The activities of SOD, POD and GSH content in *S. obliquus* were stimulated significant after treatment with Al and low pH. The results of a one-way ANOVA executed for SOD, POD, GSH and protein in *S. obliquus* treated with aluminum and low pH are shown in Table 1.

Table 1. Results of ANOVA (*F* values) executed for SOD, POD, GSH and protein in *S. obliquus* (n=15) treated with aluminum and low pH

Response variable	Al Concentration (mg L^{-1})	Source of variation		
		pH (df =2)	Time (df =4)	pH*time (df=7)
SOD	0.25	28.66	21.06	12.61
	0.70	33.31	26.81	11.24
	1.40	36.26	23.26	11.62
POD	0.25	22.42	16.25	0.64 (*P*=0.71)
	0.70	118.00	91.93	0.66 (*P*=0.70)
	1.40	50.99	40.48	6.69
GSH	0.25	51.02	19.13	0.16 (*P*=0.99)
	0.70	67.38	63.37	2.11 (*P*=0.07)
	1.40	114.03	8.29	3.67
Protein	0.25	18.52	11.67	2.92
	0.70	118.16	91.93	10.33
	1.40	50.99	40.48	4.69

The activities increased with Al concentration and acidity pH suggesting responsiveness of these enzymes to the oxyradicals generated by Al. The results are in agreement with similar findings of Kong et al. (1999) who showed that the activities of these enzymes were highest at higher Al concentration and acidic pH. Apparently, increase in the activities of distinct enzymes seems to be a rather general response to phytotoxic doses of metals, in this case aluminum. SOD and POD are enzymatic constituents of the antioxidant system and GSH is a non-enzymatic constituent formed as a result of the reduction of the oxidized form of GSH, glutathione disulfide (GSSG) by the enzyme glutathione reductase to reduced GSH (Scandalios 1997). SOD firstly catalyzes the $O_2^{\bullet}$, the fundamental free radical to H_2O_2 and then POD and GSH sequentially converts H_2O_2 to harmless O_2 and H_2O using electron donors. Scavenging of H_2O_2 by peroxide is effective even at low concentration of the enzyme, although it requires electron donors and also a regeneration system of the electron donors. This catalyzing sequence was confirmed by the present research. Compared with the initial effect of Al and low pH on SOD, the activity of this enzyme was increased about 3-4 fold after 96 hrs of treatment. Moreover, the activity of SOD was increased from 0.29 to 2.0 U min^{-1} mg^{-1} protein in the algal cells treated with 1.4 mg L^{-1} Al and pH 4.5 for 96 hrs. On the other hand, the POD activity increased only 1-2 fold with the same treatment for 96 hrs. It is clear that SOD activity was induced more than POD during time of exposure. GSH can remove the H_2O_2 generated via the oxygen free radical pathway, which is produced by the reductants autoxidizing, especially in the presence of some metals. GSH may thereby prevent the injurious consequences of the environmental conditions leading to exposure of plant cells to oxidative stress (Smith et al. 1999). In the present study, activity of the antioxidant enzymes was correlated with Al and lower pH stress in *S. obliquus*.

The enhanced level of GSH content was more pronounced at a lower pH of 4.5. Thus the previous observations substantially support the present results that elevated activities of the antioxidant enzymes protect the algae from oxidative (environmental) stress produced by Al and lower pH. It also shows that the capacity of the scavenging system is gradually increased since these enzymes showed higher activities at different phases when algae were treated with Al and low pH. The results are shown in Table 2 for the data analyzed by a two way ANOVA executed for the combination between the Al /pH treatment by time course for SOD, POD, GSH and protein in *S. obliquus*.

Table 2. Results of ANOVA (*F* values) executed for SOD, POD, GSH and protein overall in *S. obliquus* (n=15) treated with aluminum and low pH

Response variable	Source of variation						
	pH (df=2)	Al (df=2)	Time (df=5)	pH*Al (df=4)	pH*time (df=8)	Time* Al (df=8)	Time*pH*Al (df=14)
SOD	278.28	453.37	349.10	88.24	27.44	62.97	10.31
POD	183.99	530.29	370.63	22.97	5.24	45.89	4.69
GSH	414.7	138.97	189.84	74.51	3.254	23.57	1.19 (P=0.29)
Protein	153.36	28.89	18.21	32.07	6.19	113.62	8.31

It is clear that the interactions between Al concentration and pH treatments were significant and the effects of these two factors on the activity of these enzymes in the algal cells were additive effect. Moreover, the action of Al concentration treatment on the algal cells was more pronounced than that of the acidic pH treatment through the time course.

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