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Physiological and Biochemical Response of *Scenedsmus obliquus* to Combined Effects of Al, Ca, and Low pH

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The acidic precipitation caused by atmospheric pollution increases the availability of metals in aquatic ecosystem. Aluminium is one of the most important growth-limiting factors for plants and algae in acidic water (Taylor 1989). The combined toxicity of aluminium at low pH values has received considerable attention in acidic precipitation studies. Martin (1987) demonstrated that the availability and toxicity of Al are regulated by pH and the simultaneous presence of complexing ligands such as fluorides, phosphates, silicates, and carboxylates.

Algae play an important role in primary production of aquatic ecosystem. Acid rain can affect the structure and function of aquatic communities through the alterations of the species composition of an algal community. The complex toxicological problem has recently aroused considerable interest among phycologists, with efforts focused on gaining a better understanding of the toxicity mechanism of acidic precipitation on algae. Almer and others (1978) noted the paucity of green algae in lakes below pH 5.0. Despite the reduction in phytoplankton diversity, the available evidence indicates that production and biomass are comparable in acidic and nonacidic lakes, if phosphorus concentration is similar. Phytoplankton biomass was correlated with phosphorus concentration, and precipitation of phosphate by aluminium has postulated as a cause of the low phosphorus concentration in acidic lakes. The possible mechanisms by which Al might disrupt cellular functions have been proposed; one of these is that Al may induce mineral deficiencies. It was demonstrated that P nutrition can be altered by Ca and Al concentration in the environment and that Ca has a protective effect on Al and pH toxicity (Truman et al. 1986). There is little further information, however, on the physiological and biochemical responses of algae to aluminium and calcium at low pH.

Recently, the use and development of biomarkers have become of major interest in order to assess the risks of exposure to potentially toxic chemicals (John and Lee, 1990; Bucheli and Fent, 1995). The initial effects of a toxic compound on an

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organism are displayed normally as changes at the biochemical level of cell function prior to visible morphological alterations appear. It is reasonable to hypothesize that the nutrient deficiency induced by Al could be the result of the inhibition of the activity of some enzymes that were associated with the metabolism of these nutrients. Since monitoring of the biochemical response, such as the activity of these enzymes, may provide early warning signals before morbidity or mortality is visible in a population, it is possible that the effect of pollution on cellular metabolism may, therefore, be considered to be reliable indicators to monitor the level of acidic precipitation in the environment.

The objective of this study was to investigate the physiological and biochemical response of green alga *S. obliquus* to combined effects of Al, Ca and low pH. After exposure to various Al concentrations and ratios of Ca to Al at low pH, growth and protein concentrations in algal cells were measured. From measurements of acid phosphatase and nitrate reductase, the enzymes associated with the nutrient utilization of P and N; a possible mechanism of Al and low pH toxicity on algae is discussed. Additionally, the level of reduced glutathione (GSH), the predominant form of glutathinone in chloroplasts of algal cells (Chen et al., 1991), was measured. The level of GSH is a non-specific and sensitive method for the detection of the presence of bulky hydrophobic adducts. It can be altered by chemical contaminants or their metabolites that either react directly with GSH or the GSH- metabolism. However, the previous documents were focused on the varies of GSH in aquatic animals; there is relatively little attention on that in the algae.

MATERIALS AND METHODS

S. obliquus Kutz was obtained from Institute of Hydrobiology, the Chinese Academic of Science, cultured in 80 mL liquid HB-4 medium (Li, 1959) in 250 mL flasks kept on a rotator shaker at 25 °C, and illuminated with cool-white fluorescent lights (75 µmol photon m^{-2} s^{-1}) at 14:10 LD cycle. The culture medium was sterilized at 121 °C, 1.05 kg cm^{-2} for 30 min.

The pH treatments were done by adjusting the pH of medium with 1 mol/L HCl to pH 6.8 and 5.2 before autoclaving. Aluminium [as $Al_2(SO_4)3$] and HCl was incorporated into medium to get the expected concentrations of Al_3^+ (0.0, 0.25, 0.45, 0.8, and 1.4 mg/L) and pH values. The flasks were inoculated with 1 mL of fresh algal cells and incubated on the shaker for 4 d. At the end of experiment, the algal cells contained in each flask were collected by centrifuging, frozen in liquid nitrogen, and then dried and kept under vacuum at -30 °C for 14 d before each was weighed. Four replicates were set up for each treatment.

In another experiment, phosphate and nitrogen starvation were created by incubation of algal cells in medium lacking P and N before the treatment. After P and N starvation (72 hr), the algal cells were exposed to culture medium at the two pHs for each of the Ca/Al ratios. The beginning density of algal cell was 3.0 - 5.0 $x10^6$ cells L^{-1}. Calcium was added after the median effective concentration of Al (EC_{50} 0.7 mg L^{-1}) was determined according to Donald (1985). The molar Ca/Al ratios were 0/0, 0/1, 0.5/1, 1/1, and 2/1. The algal cells were inoculated for 4 d. The dry weight per unit volume algal suspension was determined by centrifuging the algal suspension, followed by a wash with distilled water and drying to constant weight at 105 °C for 10 hr.

For enzyme assays, the algal cells were collected and extracted with 1 mL of Tris/Borate (0.1 M / 0.3 M, pH 7.5, 5 mM EDTA, and 7 mM β-mercaptoethanol) buffer on ice for 10 min. The extract was then centrifuged at 10,000 x g for 10 min at 4 °C, the extracts were stored at -58 °C until the enzyme were measured (Kong and Chen, 1995).

Nitrate reductase (NR, EC. 1. 6. 6. 2) was measured by the method of Hageman (1980). Acid phosphatase (AP, EC 3. 1. 3. 2) was assayed by measuring the liberation of p-nitrophenyl from substrates, PNP-phosphate, at 405 nm (Boller and Kende, 1979).

For GSH assay, the algal cells were treated with Al [as $Al_2(SO_4)_3$ and $AlCl_3$ respectively] and Ca/Al. The theoretical concentrations of $A1^{3+}$ and the ratios were the same as mentioned above. After the treatments, the algal cells were collected and dried and the concentrations of GSH were determined according to Zhang et al. (1990). Briefly, the algal cells were homogenized in 1 mM EDTA and trichloroacetic acid (the end concentration was 0.3 M). GSH was measured using 300 µl of the supernatant. To the supernatant, 1.5 mL of phosphate/EDTA buffer (125 mM phosphate, 6.3 mM EDTA buffer pH 8.0) and 0.15 mL phthalaldehyde dissolved in methanol (1%) were added. The absorbance of the solution was read in a spectrofluorometer at 343 nm (excitation) and 425 nm (emission) after 1 min incubation.

Protein concentrations in the algal cells were determined by the method of Lowry et al. (1951) and with bovine serum albumin (BSA) as the standard. Enzyme activities were expressed in mU per mg soluble protein.

All biochemical compounds used in this experiment were from Sigma (USA). Statistical analyses were performed using the Statgraphics software package.

RESULTS AND DISCUSSION

Growth of the alga was monitored by measuring the variation in dry weight of the algal cells per unit volume of culture suspension. The growth was inhibited by higher concentration of aluminium in this experiment ($p < 0.01$). *S. obliquus* showed about 50% of growth compared with the control at 0.7 mg L^{-1} at pH 6.8 and 5.2 ($p < 0.01$, Fig. 1A). There was no statistically significant effect of pH values ($p > 0.05$) on the algal growth. When Ca was added to the culture medium, the response of the algal cells to aluminium was not significantly changed ($p > 0.05$).

However, an increase in the soluble protein concentration in the cells treated with Al was observed (Fig. 1B). When the concentration of Al at 0.7 mg L^{-1} was applied, the protein concentration is higher than that in the control (0.0 mg L^{-1}) in both pH 5.2 and 6.8. Previous reports on the effect of metals on the protein concentration vary. Our results were in agreement with the findings of Assche et al. (1988) who showed that protein concentrations increased in roots and leaves of plants after application of metals, such as Cu, Al and Zn. In our experiment concerning the effects of Al and Zn on another unicellular green alga *Seleastrum capricornutum* (Kong and Chen, 1995) the protein concentrations in cells showed similar tendency as that in *S. obliquus*. There was no significant difference among the treatments with the various ratios of Ca/Al ($p > 0.05$).

Acid phosphatase, an important enzyme associated with P utilization, was dramatically reduced by the application of Al (Fig. 1C); A decrease in AP activity was observed when Al was added (Ca/Al was 0/1, $p < 0.01$); AP showed only 50% activity, relative to that in control at pH 5.2 and 6.8. AP activity was induced after calcium was added to culture medium in both pH 5.2 and 6.8. The activity was highest when Ca/Al was 1/1, although it was lower than that in the control. Early studies suggest that Al might induce mineral nutrient deficiencies that form the basis of Al toxicity. Some of evidences was also used to support the hypothesis that the toxic effects of Al could be the result of an Al-induced P deficiency. For example, the supply of P to cultural medium has a protective effect against Al injury (Bennet et al. 1987). The present experimental data provides direct evidence to support this hypothesis. It is notable that an increase in activity of AP seems to be due to an increase of the ratio of Ca/Al. The addition of Ca has increased AP activity. Clearly, the variation in acid phosphatase activity in the algal cells may be related to the utilization of phosphorus. In the other hand, pH values tested in this experiment had no statistically significant effect on this enzyme ($p > 0.05$).

Aluminium stress also disrupts N assimilation. Previous documents reported

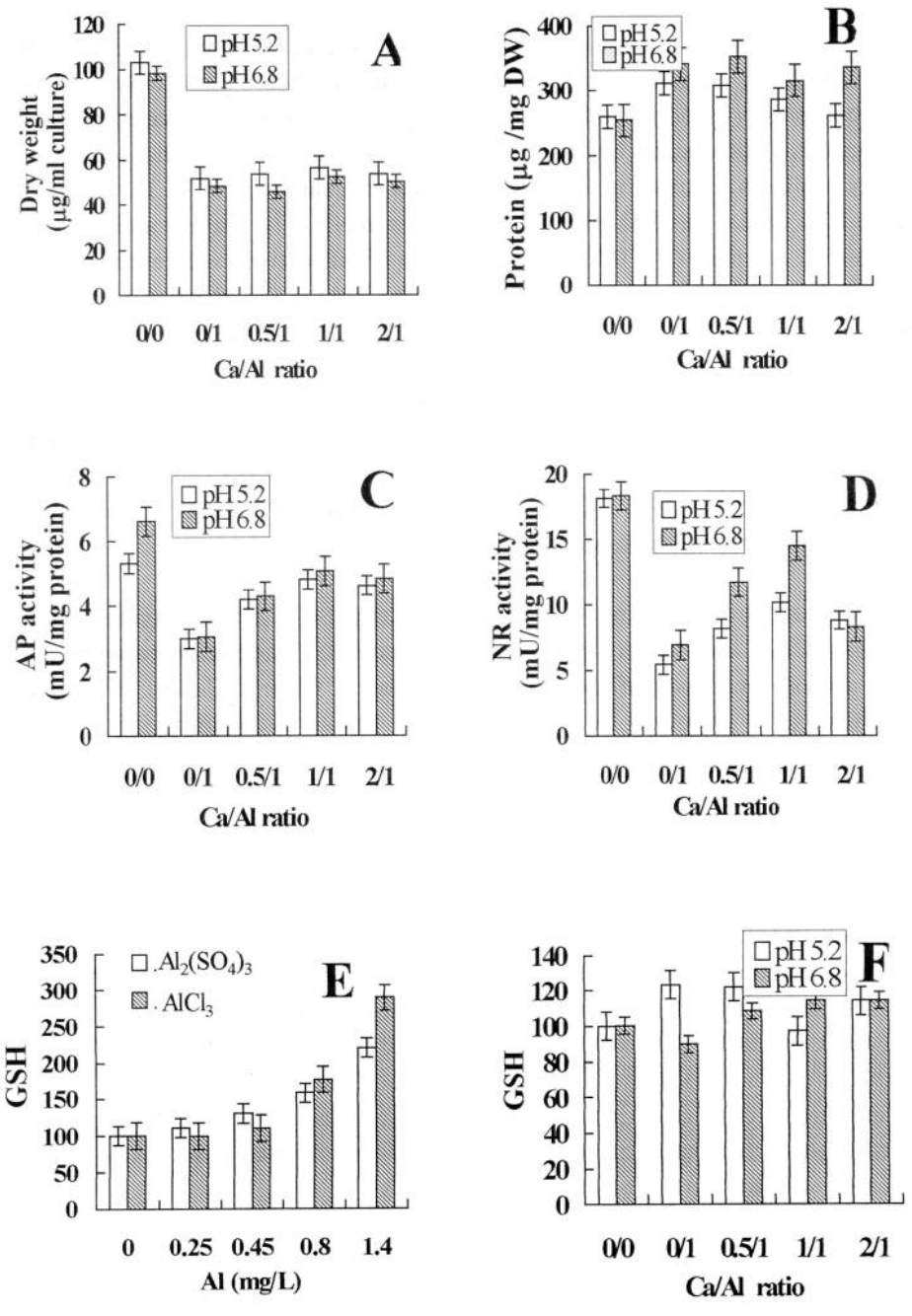

Figure 1. Responses of *S. obliquus* to Al, Ca and low pH

that Al reduced NO_3^- assimilation in some plant whereas in other species, both NO_3^- and NH_4^+ uptake were reduced. Reduced nitrate reductase activity could be partially responsible for reduced NO_3- assimilation under conditions of Al stress, An association between tolerance to Al and high nitrate reductase activity has been reported in *Triticun aestivum* (Fernandez, 1989). In the present study, a similar trend like for AP was shown in NR. The enzyme activity was inhibited by Al stress ($p < 0.01$). Measurement of NR activity indicated that the enzyme showed 37 % and 30 % activity in the Ca/Al at 0/1 at pH 5.2 and 6.8, respectively, relative to the control. However, with the increase of Ca/Al ratios from 0/1 to 1/1, this enzyme activity was induced from 6.91 to 14.52 and 5.44 to 10.16 mU/mg protein at pH 5.2 and 6.8 respectively. These results suggested that Al had a toxicity to this enzyme and Ca had protected the NR activity from the Al injury ($p < 0.01$). Our results are in agreement with the findings of Keltjens and Van Ulden (1987) who suggested that low pH values and Al-stress disrupted NO_3^- assimilation in plants.

A number of reports dealing with Al and lower pH toxicity to organisms are available. Wood and others (1984) reported a complex interaction between Al, Ca, P, and pH on growth of plant (*Trifolium repens*). Clearly, Ca and phosphate had a protective effect on Al and pH toxicity. Under the conditions of acidic solutions regarding pH, Ca, and Al concentrations, the Al stress is strongly governed by the Ca/Al ratios (Ulrich, 1989). Such ratios represent the relation in the chemical potential of the ions involved to be adsorbed at negatively charged surfaces like the cell wall and cell membranes. This adsorption is the first step in a series of chemical and biochemical reactions resulting in an effect. This makes the usefulness of such ratios understandable. Eldhurst and others (1987) also found that Al can reduce the uptake of Ca and the reduction in plant growth and damage seems to be regulated mainly by the Ca/Al ratio. Concentrations of Ca in plants of numerous species have been shown to be reduced by Al stress. Furthermore, increased supply of Ca to Al-toxic growth solutions decreased the extent of Al injury. These investigations and the present study suggest that this effect might be due in part to effects of ionic adsorption on the speciation of Al.

The studies with aquatic organisms have shown that the levels of GSH are responsive to exposure to contaminated environment (Stein et al., 1992). An important feature of GSH is its inducibility under conditions of oxidative stress. GSH can remove the H_2O_2 generated via the oxygen free radicals, which is produced by the reductants autoxidize, especially in the presence of some metals. In the present study, the elevated concentration of GSH with the increase of Al concentration was noteworthy ($p < 0.01$, Fig. 1E). The concentrations of GSH in treatments with 1.4 mg L^{-1} Al were about 2- [$Al_2(SO_4)_3$] or 3-fold

($AlCl_3$) that in the control. In addition, the GSH showed the highest level in the treatment with Ca/Al of 0/1 relative to the other treatments (Fig. 1F). In other words, the GSH concentration decreased with the addition of Ca ($p < 0.05$). Because a decrease of GSH had been related to a saturation of the cellular capacity to detoxicate reactive xenobiotic pollutants, it is further supported from the another point of view that the addition of Ca really plays a role to reduce the Al and acidic stress on the algae. On the other hand, there were no a notable relationship between the GSH level and the ratios of Ca to Al at pH 6.8. It is possible that the availability of Al was not in agreement with the Al concentration in medium at this pH value. These observations suggest that oxidative stress is involved in processes which resist oxidative damage within lipid membranes of cells caused by Al stress. Although further studies are needed to delineate the consequences of elevated GSH in algal cells, the results of the present study suggest that GSH represents a promising bioindicator to Al stress. Moreover, due to the role of GSH in the defence against toxicity, the increase of GSH may represent an adaptive response to contaminant exposure. Though it is unknown whether the increase in GSH is solely an adaptive response to contaminant exposure, the GSH level in organisms can be used to early monitor the effects of chemical compounds on environment.

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