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Role of Proline Accumulation in Response to Toxic Copper in *Microcystis aeruginosa*

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The blue green alga, *Microcystis aeruginosa,* was found to accumulate proline under the stressful concentration of cupric ions. The changes of proline level in *Microcystis aeruginosa* in response to copper(Cu) have been monitored and the function of the accumulated proline was studied with respect to its effect on Cu uptake. Exposure of *Microcystis aeruginosa* to elevated concentrations of Cu led to accumulation of free proline depending on the concentrations of the metal in the external medium. The greater the toxicity or accumulation of the metal, the higher the amount of proline in algal cells were found. When proline was exogenously supplied prior to Cu treatment, the absorption of Cu was markedly reduced. When exogenous proline was supplied after Cu treatment, it resulted in a remarkable desorption of the adsorbed Cu immediately after the addition of proline. Pretreatment of *Microcystis aeruginosa* with proline counteracted with metal-induced lipid peroxidation. The results of the present study showed a protective effect of proline on metal toxicity through inhibition of lipid peroxidation and suggested that the accumulation of proline may be related to the tolerance mechanism for dealing with Cu stress.

Key words : copper, *Microcystis aeruginosa*, Cu uptake, proline accumulation, lipid peroxidation

1. Introduction

Algal cells have remarkable ability to take up and accumulate heavy metals from their external environment. High concentration of metals, including those essential for growth and metabolism, exert toxic effects on the metabolic machinery of algae. The interactions between algae and metals involve the adsorption of the metal ions on their surfaces and intracellular uptake[1,2]. Algae may become tolerant to heavy metals through various mechanisms, such as intracellular binding of metal ions, extracellular exclusion of metal ions or a reduction of uptake of metal ions[3].

Copper is an essential element for metabolic processes in algae, being required for electron transport in photosynthesis and various enzyme systems. However, in excess copper it causes damage at the morphological, biochemical and ultrastructural levels[4]. It is also one of the most toxic trace metals to algae. Copper has been reported to inhibit photosynthesis, disrupt electron transport in photosystem Ⅱ, reduce pigment concentrations and restrict growth[5,6]. It has been suggested that Cu affect cell motility[7] and the distribution of other compounds such as proteins, lipid, sterols, sterol esters and free fatty acid[8]. It was found that Cu adsorbed more efficiently than other metals(Zn, Mn and Cd) to the cell walls of algae[9]. Algae have been shown to employ several mechanisms to tolerate copper toxicity. Tolerance can occur either through exclusion mechanisms, for example, by production of metal binding compounds, extracellularly, or associated with the cell wall, or through intracellular detoxication[10].

Proline accumulation is found in a variety of organisms, including algae, bacteria and higher plants and takes place in response to stressful condition such as salinity, drought, temperature shock and air pollution. Numerous studies have shown that the amino acid proline accumulated in microalgae in response to elevated salinity[11].

Accumulation of free proline in response to heavy metal exposure seems to be wide-spread among plants[12]. Previous studies reported that the accumulation of proline can also be detected in some algal cells during contact with deleterious concentrations of copper[13,14]. However, the survival value of proline accumulation under heavy metal stress has been a matter of debate. Numerous studies have shown that proline accumulation is a symptom of injury which does not confer tolerance against metal or other stresses[15]. Schat *et al.*(1997)[12] observed that metal-induced proline accumulation did not occur until the damage had been caused and consequently, it did not apparently prevent metal toxicity. By contrast, suggestions have been made that proline might protect plants from heavy metal toxicity[16]. Nevertheless, it is still unknown whether proline accumulation is related to metal tolerance. Thus it is of great interest to study the possible role of proline accumulation in the stressed algal cells.

Another distinctive point is that diverse kinds of stresses including heavy metals induce lipid peroxidation, which leads to disruption of membrane functions and harmful effects on plant cells[17]. There appears to be a relationship between lipid peroxidation and proline accumulation in plants subjected to diverse kinds of stresses. If such a relationship exists, proline accumulation might have a role in inhibiting metal-induced lipid peroxidation.

The present study focuses on the effect of proline on the uptake of Cu by the blue green alga *Microcystis aeruginosa,* a common species producing nuisance blooms in lakes or rivers. The kinetics of proline accumulation, the relationship between intracellular contents of metal and proline, and the protective role of proline were investigated.

2. Materials and Methods

2.1. Algal culture conditions and growth

Stock cultures of *Microcystis aeruginosa* UTEX 2388 were grown axenically in modified Allen's medium : $NaNO_3$(17645.0 μM) ; K_2HPO_4(223.9 μM) ; $MgSO_4 \cdot 7H_2O$(304.3 μM) ; Na_2CO_3 (200.0 μM) ; $CaCl_2$(243.3 μM) ; $Na_2SiO_3 \cdot 9H_2O$ (204.1 μM) ; ferric citrate(24.5 μM) ; citric acid (31.2 μM) ; EDTA(3.4 μM) ; H_3BO_3(46.2 μM) ; $MnCl_2 \cdot 4H_2O$(9.2 μM) ; $ZnSO_4 \cdot 7H_2O$(0.8 μM) ; $Na_2MoO_4 \cdot 2H_2O$(1.6 μM) ; $CuSO_4 \cdot 5H_2O$(0.3 μM) ; $Co(NO_3)_2 \cdot 6H_2O$(0.2 μM). The growth medium(pH 7.8 with 0.1 N HCl) was prepared in deionized water. Cultures were grown in 250 ml Erlenmeyer flasks in a growth chamber with 16 h light(50 μmol $m^{-2}s^{-1}$) : 8 h dark cycle, at 28 ± 1℃. The cultures were hand-shaken two times daily. Algal biomass was recorded as absorbance at 685 nm, and the specific growth rate, based on absorbance, was calculated for the control and treatments[18].

Growth rate ; $dX / dt = \mu X$

where X ; number of cells, μ ; growth rate, t ; time

$\mu = \ln X_2 - \ln X_1 / t_2 - t_1$

T(doubling day) = ln2(0.6931) / μ

2.2. Uptake of Cu

The time-course of the metal uptake was studied at the concentrations of metals causing ca. 50 % inhibition of the growth rate. The cultures were treated with 5, 10, 20, 30 and 40 μM of Cu. Stock solutions of test metal were prepared from $CuSO_4 \cdot 5H_2O$. Samples were taken at various time intervals to determine the intracellular metal content, and for cell counting with a haematocytometer. The samples were then centrifuged to harvest the cells. The algal pellet was washed in distilled water to remove surface-bound metal. After centrifugation, the pellet transferred to 5 ml of a digestion mixture containing HNO_3(70 %). Digestion was performed on a hot plate at 80℃ until the solution became colourless. The final volume adjusted to 10 ml. The samples were analysed for metal content with a ICP atomic emission spectrometer(Thermo Jarrell Ash, ICP-IRIS, U.S.A.). To ascertain the toxicity of Cu-proline complex, the concentrations of exogenously supplied proline and Cu were adjusted. For these experiments, the proline was added to the culture media prior to the addition to the addition of Cu. The growth rate and the uptake of Cu by the cells were measured after an incubation of 24 h.

2.3. Measurement of proline content

Proline content in the cell extracts was determined according to the method described by

Bates *et al.*(1973)[19]. Cell pellets were washed by centrifugation at × 1,000 *g* for 10 min and stored at -70℃ for further analysis. The algal pellet was resuspended in 10 ml of 3 %(v/v) sulfosalicylic acid and cells were disrupted with an ultrasonicator for 10 min at 300 mA. Two ml of the extract was treated with 2 ml of acid-ninhydrin and 2 ml of glacial acetic acid in a test tube for 1 h at 100 ℃ and the reaction terminated in an ice bath. The reaction mixture was extracted with 5 ml toluene and mixed vigorously for 15-20 sec. The chromphore containing toluene was separated and the absorbance was read at 520 nm. The proline concentration was then calculated on a dry weight basis.

2.4. Measurement of lipid peroxidation

The level of lipid peroxidation was measured in terms of malondialdehyde(MDA) content using the thiobarbituric acid reaction[20]. The pellet was mixed in 0.1 %(w/v) trichloroacetic acid(TCA). Five ml of 20 %(w/v) TCA containing 0.5 %(w/v) thiobarbituric acid was added to pellet. The mixture was heated at 95 ℃ for 40 min and then rapidly cooled in an ice-bath. After centrifugation at × 10,000 *g* for 10 min, absorbance of the supernatant at 532 nm was read and the value for the non-specific absorption at 600 nm was subtracted. The concentration of MDA was calculated using its extinction coefficient of 155 $mM^{-1}cm^{-1}$. All the experiments were conducted at least twice, with triplicate measurements for each treatment.

3. Results and Discussion

To study the possible role of proline enhancement of growth, exogenous proline was supplied to the cell cultures, and the changes in the growth rate of cells from Cu-treated cells and proline content in cells were examined(Fig. 1). In the absence of deleterious concentrations of Cu, proline slightly enhanced the growth of cells. Fig. 2 shows the growth rate of algal cells according to the various concentrations of Cu. When the cultures were treated with 5, 10, 20, 30 and 40 μM of Cu, inhibition of cell growth was observed at concentrations above 10 μM Cu. Exogenous proline(0.1 mM) supplied to algal cultures lowered the inhibitory effect of Cu. The 40 % reduction of cell growth was observed in 20 μM Cu-treated cells. There was no increased cell growth above 40 μM Cu. The effect of proline on lowering the Cu toxicity was varied with the Cu concentrations tested.

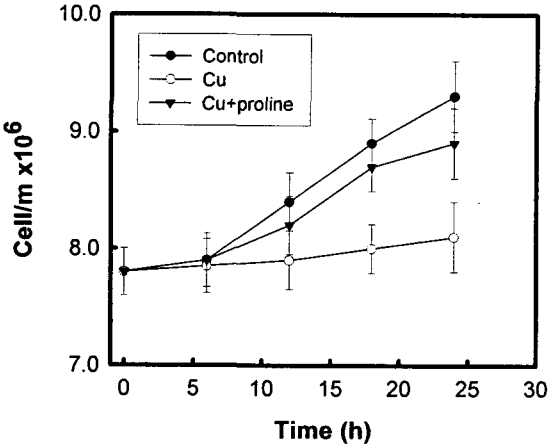

Fig. 1. Growth patterns of *M. aeruginosa* exposed to Cu(20 μM) in the presence and absence of exogenous proline(0.1mM).

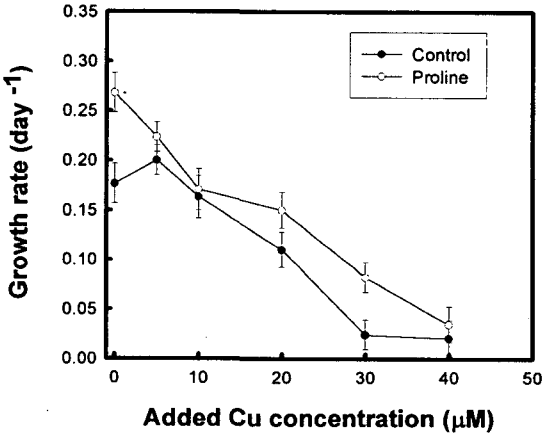

Fig. 2. Growth rate of *M. aeruginosa* treated with various concentrations of Cu in presence and absence of exogenous proline(0.1mM).

The growth rate of cells at various concentrations of exogenous proline was slightly increased to 0.12 day^{-1} exposed to 20 μM Cu in 0.01 mM and 0.1 mM proline-treated cells(Fig. 3). However, the growth rate of cells showed a significant increase about three times when supplied 1 mM proline. Proline alleviated the inhibitory effect of Cu in a concentration-depending manner. In contrast,

proline content of cells at various concentrations of exogenous proline showed quite different pattern. Proline content of 1 mM proline-treated cells was lower than that of 0.1 mM proline-treated cells.

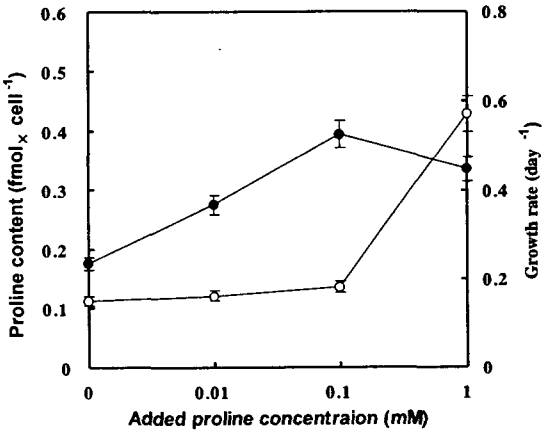

Fig. 3. Changes in the growth rate(○) and intracellular proline content(●) of *M. aeruginosa*. cells at various concentrations of exogenous proline. All media were exposed to 20 μM Cu.

Fig. 4 shows time-course of metal uptake and proline accumulation in *M. aeruginosa* exposed to 20 μM Cu and 0.1 mM proline, independently or in combination. Uptake of Cu rapidly increased in the initial stage of incubation(Fig. 4A). Cu uptake in Cu-treated cells increased to seventy times of initial value after 6 h incubation, whereas proline-treated cells was increased to sixty times of initial value. Maximum value was detected after 12 h incubation in Cu treated cell.

There was a sharp rise in intracellular proline content after 6 h exposure to Cu(Fig 4B). The rise in proline content of Cu-treated cells was 14 times of control. After reaching the peak, the concentration of proline in Cu-treated cells decreased rapidly in the next 18 h incubation, and then slowed until the end of the experiment. The concentration of proline in the Cu-treated cells in combination with proline gradually increased during 24 h incubation. Many studies suggest that intracellularly accumulated proline is related to the tolerance to Cu in algal cells[13,14,21]. In addition to the species reported previously, *Microcystis* also accumulated proline intracellularly in response to Cu stress. This allows a comparative study of the role of proline in cells containing different intracellular proline levels. Regarding the tolerance to metals, a variety of mechanisms have been reported for algae ; exclusion from cells[22], metal-binding peptides and proteins[23], binding and precipitation within the cytoplasm and or vacuole[3] and sequestration with electron-dense polyphosphate granules[24]. Cu-induced enhancement of the concentration of intracellular proline was also reported in higher plants[25] and other algae[14,21]. The pattern and magnitude in algae, however, were different when compared with higher plants. The present work thus shows a close link between the process of Cu uptake and proline accumulation.

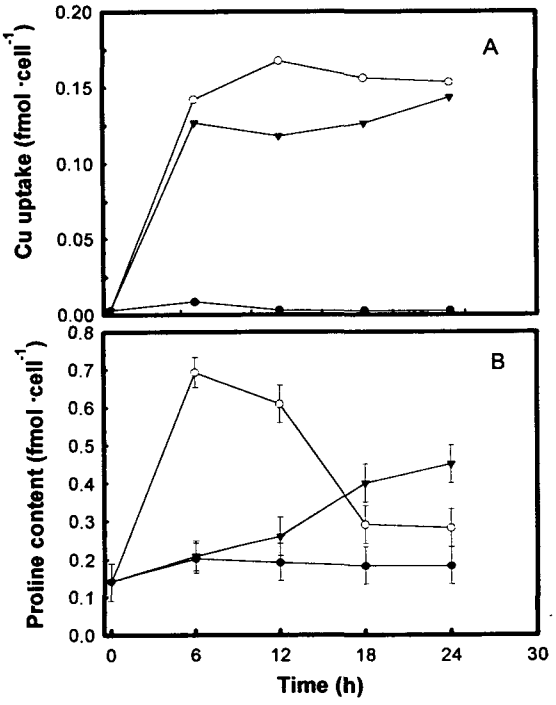

Fig. 4. Time-course of Cu uptake(A) and proline accumulation(B) by *M. aeruginosa* cells with Cu(20 μM) and proline(0.1mM) alone or in combination. ● , control ; ○ , Cu ; ▲ , Cu+ proline.

To study further the effect of proline on Cu uptake, exogenous proline was supplied to the culture medium. The addition of proline (0.1 mM) to algal cultures was made alone with Cu. As the

concentration of Cu in the external medium increased, there was a concomitant increase in the amount of Cu taken up by the cells. Proline treatment in the presence of Cu, was effective in enhancing Cu uptake by the cells(Fig. 5). It is clear that proline reduces the uptake of Cu compared with the control. During the exposure to deleterious concentrations of Cu, the level of intracellular proline increased as Cu increased from 5 to 30 μM, but it was low at 40 μM Cu(Fig. 6).

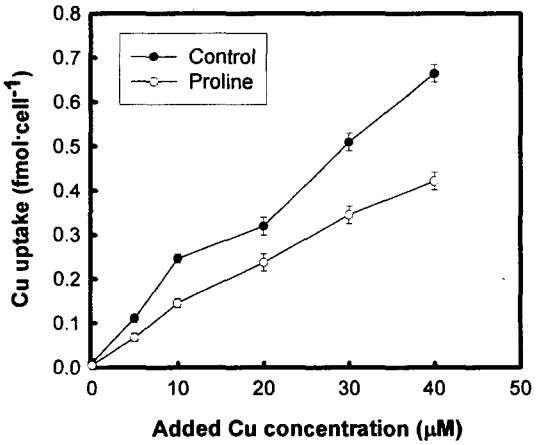

Fig. 5. Effect of the preincubation in the culture medium containing 0.1 mM proline on the uptake of various concentrations of Cu by *M. aeruginosa* cells.

Proline has an appreciable affinity to forming various complexes with cupric ions[26], which may be partly attributable to the reduction in Cu toxicity. The present study shows that increasing amounts of intracellular proline reduces the internalization of Cu from the external medium in *Microcystis* sp. This could result from either enhanced efflux of Cu from the cytoplasm or reduced absorption of Cu. In the former case, it is assumed that the efflux of Cu is enhanced when it is complexed by proline. This effect may be ascribed to the complexation between proline and Cu and the subsequent displacement of the sorption equilibrium between Cu in culture medium and Cu adsorbed on the cell surface.

Fig. 7 shows the relationship between intracellular concentrations of proline and Cu uptake. The amount of Cu taken up by the algal cells is positively correlated with the intracellular proline

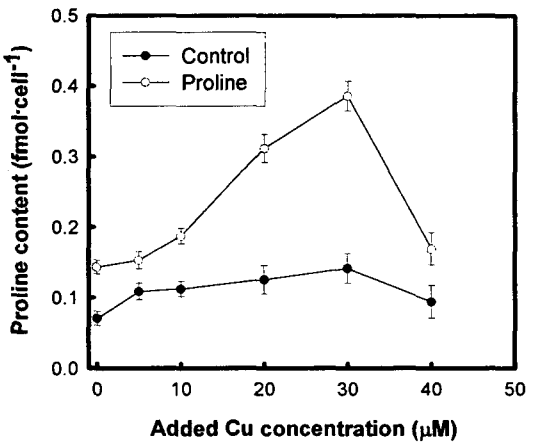

Fig. 6. Changes in the proline content of *M. aeruginosa* cells at various concentrations of exogenous Cu.

level. When proline was supplied prior to Cu treatment as shown in Table 1, the amount of Cu taken up by the algal cells was markedly reduced when compared with control. The reduction in Cu uptake was observed either in the medium in which Cu was nearly completely complexed or in those in which Cu was only partially complexed by added proline. The present observations showing a positive relationship between metal toxicity and proline accumulation suggest a protective role of this amino acid against heavy metal toxicity. In this content, suggestions have been made that proline provides protection by ; maintaining the water balance which is often disturbed by heavy metals[12,27], scavenging hydroxyl radical[28], chelating heavy metals in the cytoplasm[29], and reducing metal uptake[14]. Many researchers, nevertheless, feel that proline accumulation is merely a symptom of diverse stresses, and is not involved in protection against metal and other stresses[15]. Proline pretreatment provided protective role by reducing Cu uptake by *M. aeruginosa.* It might be that proline reduces the production of harmful radicals, or sequesters them. Alternatively, proline might complex metal ions inside the cell thereby protecting sensitive cellular sites from toxic effects.

To study the effect of proline on lipid peroxidation, which leads to disruption of membrane functions and harmful effects on plant cells, the relationship was examined between lipid peroxidation and proline accumulation in *M. aeruginosa*

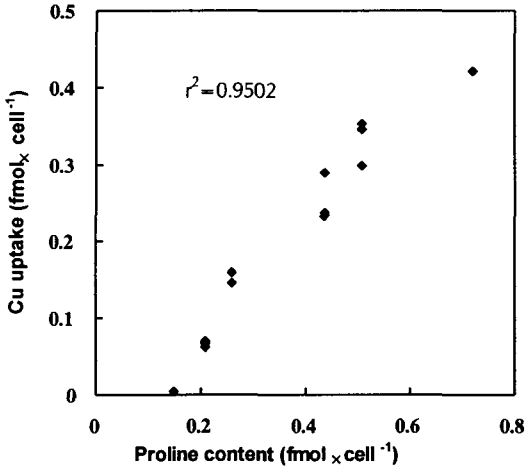

Fig. 7. Plot of the uptake of Cu at various concentrations(5, 10, 20, 30 and 40 μM) by *M. aeruginosa* cells containing various intracellular proline levels.

Table 1. Effect of proline addition in *M. aeruginosa* cells supplied either prior to or after Cu (20 μM)

Treatment	Proline(mM)	Cu uptake (fmol • cell^{-1})
Control	0	0.25±0.02
Pretreatment	0.1	0.77±0.05
	1.0	0.65±0.05
Treatment after	0.1	1.40±0.09
Cu exposure	1.0	1.30±0.07

cells subjected to various concentrations of Cu. Lipid peroxidation was markedly increased when algal cells were exposed to test metal(Table 2), indicating a strong inducer of lipid peroxidation. Pretreatment of proline substantially reduced lipid peroxidation even in the control. Proline pretreatment mitigated metal-induced lipid peroxidation in *M. aeruginosa* in a concentration-dependent manner. There is at least one similarity in the mode of action of heavy metals, air pollutants, UV-B radiation, drought and salinity on plants ; all of them induce oxidative stress[30,31]. Weckx and Clijsters(1996)[32] showed that Cu induces oxidative stress in primary leaves of *Phaseolus vulgaris*. Oxidative stress causes lipid peroxidation and thereby destruction of cell membranes[31]. The present work showed that Cu was a efficient inducer of lipid peroxidation in algal cells. It was shown that Cu affects the capacities of the enzyme involved in the ascorbate-glutathione cycle, suggesting that the cycle might be considered to contribute to the cellular defence against Cu-mediated oxidative stress together with enzymes quencing reactive oxygen species and/ or reducing NAD(P)$^+$[33]. Lipid peroxidation by Cu in the algae might have led to distruption of the plasma membrane thereby causing the leakage of K^+ from algal cells[13]. Proline pretreatment provided protection by reducing metal-induced lipid peroxidation. It is likely that proline acts on the cell plasmalemma and protects it from being affected by toxic Cu, suggesting that proline possibly plays a role as a protectant for the plasmalemma against Cu. There is certainly another possibility that proline lowers the toxicity of Cu via its complexing with Cu, so that the concentration of effective free Cu is decreased.

Table 2. Lipid peroxidation measured as 2-thiobarbituric acid-reactive material(TBA-rm) in *M. aeruginosa* treated with 20 μM Cu for 1 h. Proline pretreatment was given for 15 min.

Treatment	TBA-rm($A_{532-600}$ nmol/ml of dry wt.)
Control	33±4
Proline(0.1 mM)	21±5
Proline(1.0 mM)	15±5
Cu(20μ M)	95±12
Cu+proline(0.1 mM)	56±9
Cu+proline(1.0 mM)	27±6

It has been acertained that the cell plasmalemma is the primary target of the toxic action of trace and heavy metals. Proline is capable of inhibiting the leakage of potassium ions, the symptom of damage in the plasmalemma from Cu-treated *Anacystis nidulans* cells, suggesting that proline is one of the compatible solutes that have the function of stabilizing the folded protein structure and therefore influence protein solvation[34]. Zhao *et al.*(1992)[35] speculated that compatible solutes might protect the membrane integrity and affect the stability of membrane. On the basis of these

facts, it is assumed that the physiological role played by intracellularly accumulated proline, in addition to forming complexed with Cu, is possibly to protect the cell membranes by stabilizing plasmalemma permeability. It might be that proline reduces the production of harmful radicles, or sequesters them. Alternatively, proline might complex metal ions inside the cell thereby protecting sensitive cellular sites from toxic effects. Since the survival value of proline accumulation under heavy metal stress has been a matter of debate, it is necessary to investigate in detail how proline acts on the cell wall or plasmalemma of algal cell.

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