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Effects of Heavy Metals on Growth and Protein Synthesis in Cyanobacterium *synechocystis* sp. PCC 6803

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중금속이 Cyanobacterium *synechocystis* sp. PCC 6803의 성장과 단백질 합성에 미치는 영향

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ABSTRACT

The changes of growth and protein synthesis pattern by aluminum (Al), cadmium (Cd), zinc (Zn) treatments were studied in Cyanobacterium *synechocystis* sp. PCC 6803.

When exposed to Al from 5ppm to 30ppm, *synechocystis* grows normally. But more than that retard the growth of algae notably. The 0.05ppm Cd additions had no effect on the growth of algae. 0.1, 0.2, and 0.5ppm Cd inhibited growth. Under 1 and 2ppm Cd stress, growth was greatly diminished. Zn had dual effects. The growth of algae in media containing 5ppm was stimulated. As concentration increases more than 15ppm, growth inbition increases. Under 25ppm Zn stress, growth was greatly diminished. According to logistic theory, r and K values of each heavy metal-treated groups were estimated. Correlation analysis of r and K values with metal concentration showes that there is negative correlation between K and concentration in Cd and Zn treatments. Critical concentration which shows lethal or sublethal effect was estimated by t-test of each r and K value.

The cells cultured in 10, 20, 30, 40 and 50ppm of Al, 1 and 2ppm of Cd, and 10, 15, 20, 25 and 30ppm of Zn for 4 days was used for protein analysis. Analysis of protein synthesis with SDS-PAGE showed alterations of protein synthesis pattern. The synthesis of protein about 220kD increased markedly.

In this study, it showed that resistance mechanism against Al, Cd, and Zn is K selection and that metal stress induced the change of protein synthesis in Cyanobacterium *synechocystis* sp. PCC 6803.

Key words: Cyanobacterium *synechocystis* sp. PCC 6803, Heavy metals, Aluminum, Cadmium, Zinc, Growth, Protein synthesis.

INTRODUCTION

Among environmetal pollutant, the effects of heavy metals on an organism are very important problem. In the last years, the effect of heavy metal accumulating in soil, plant, animal and microbes has been studied steadily. For microbiota, much information has been obtained. Studies using laboratory media and, usually, pure cultues of microorganisms have elucidated the relative toxicities of different metals, the comparative sensitivities of different microbial species and genera to metals, the levels of metals at which growth inhibition occurs initially and at which complete toxicity results, the subtle effects of metals on microbial growth kinetics, spore formation and germination, morphology, and biochemical activities, such as respiration and photosynthesis, the modes of resistance to metal toxicity and the influence of abiotic factors in mediating metal toxicity to microbes (Babich and Stotzky, 1980, 1982, 1983, 1985; Stoszky and Babich, 1984).

Aluminum is one of the most ubiquitous elements in the environment. It has, until recently, existed predominatly in forms not available to man and most other speicies. Acid rain, however, has increased dramatically the amount of aluminum appearing in biologic ecosystems, resulting in well-described destructible effects on fish and plant life species (Casarett & Doull, 1991). The study for biological effect of Al has been focused Al resistant plant and effect on human body. The effect on cyanobacteria, soil bacteria and mycorrhizal fungi was studied (Pina & Cervants, 1996). Cadmium is a modern toxic metal. It is a very important metal with many applications. Its main use is electroplating, galvanizing, color pigment for paints and plastics and cathode material for battery. Cd is a by-product of zinc and lead mining and smelting, which are important sources of environmental pollution (James & Ramamoorthy, 1983). Zinc is an essential element for animals and plants. More than twenty different zinc metalloenzymes have been identified. These include carbonic anhydrase, alkaline phosphatase, and alcohol dehydrogenase. Zinc plays a vital role in the biosynthesis of nucleic acids, RNA polymerase, and DNA polymerase. Thus zinc is involved in the healing processes of tissues in the body. A number of other physiological processes, including hormone metabolism, immune response, and stabilization of ribosomes and membranes, require zinc (James & Ramamoorthy, 1983). But excess of Zn has lethal effect such as other heavy metals.

The responses of plants against heavy metal are sensitivity and resistance. The past studies has been perfomed focusing sensitivity, but recent studies are focusing resistance. Therefore, the study of metal-resistant plant and mechanism has been increased. Metal-resistant plants are duckweed (David & Jennifer, 1993; Chang & Oh, 1986), *Zizania* and *Typha* (Kim and Cho, 1995), dropwrot (Lee, 1995; Na and Kwun, 1994) and isinglass (Lenka *et al.*, 1990). Some studies showed that cyanobacteria absorbs more metals than other bacteria, algae and fungi. Thus, in aquatic ecosystem, considerable amounts of Fe, Zn, Cu, Pb is removed by cyanobacterial mats (Taher *et al.*, 1994; Maquieira, 1994).

Stimulus such as heat, light, salt shock and pH change effects on protein, RNA and DNA sysnthesis in cells (Sinha *et al.*, 1996; Hagemann *et al.*, 1994; Satoh, 1996; Robinson *et al.*, 1996). In case of animals, metal induces metallothionein(MT), metal binding protein. MT stores essential element as well as heavy metal and reduces toxicity of heavy metal by binding metal when exposed to excess metal (Kille *et al.*, 1994). In case of plants, metal binding protein similiar to MT of animals was detected. But, the study about macromolecule such as DNA, RNA and protein which are induced by heavy metal in algae is deficient.

Therefore, this study showed growth and protein synthesis changes when heavy metal was added to Cyanobacterium *synechocystis* sp. PCC 6803.

MATERIALS AND METHODS

1. Organisms

Cyanobacterium *synechocysis* sp. PCC 6803 used in this study was obtained from life science laboratory of Korea Basic Sience Center in standard research institute.

2. Culture condition

According to Kraz *et al.* (1955) & Jurgens *et al.* (1985), cell culture was performed. Plate culture was performed using BG-11 containing agar(1.5%) and glucose(2%). Liquid culture was performed using medium C containing 1.5% glucose. Culture temperature was 28℃ and light intensity was 20 mol /m^2 · sec

3. Analysis of growth by heavy metal treatment

1) Stock solution

Stock solution of Al, Cd and Zn consisted of 4.9680g of $AlCl_3$, Cd 1.6305g of $CdCl_2$ · 2.5 H_2O and 2.0845g of $ZnCl_2$ per 1L of D.W, respectively.

2) Heavy metal treatment

For growth analysis, cell was cultured in medium containing 5, 10, 15, 20, 25, 30, 35, 40ppm Al, 0.05, 0.1, 0.2, 0.5, 1, 2, 3, 4ppm Cd and 5, 10, 15, 20, 25, 30, 35, 40ppm Zn. Until growth approach steady state, the absorbance was read at 730nm on spectrophotometer absorbance.

3) Growth analysis

If growth of Cyanobacterium *synechocystis* sp. PCC 6803 was analyzed using logistic equation (Pearl and Reed, 1920) which express following equations, we know which selection mechanism against heavy metal is K selection or r selection.

$$N_t = \frac{K}{1+e^{a-rt}}$$

N_t ; pupulation size at time t

t ; time

K ; maximal value of N

e ; 2.71828 (base of natural logarithms)

a ; a constant of integration defining the position of the curbe relatibe to the origin

r ; rate of population growth per capita

Results of this study were analized by using SPSS/PC$^+$. Correlation analysis and t-test was performed between heavy metal concentration and K, r value except for sample which stopped growth instantly after metal treatment.

4. Protein analysis

1) Sample preparatiion

Mass culture for protein analysis was performed in media containing 10, 20, 30, 40 and 50ppm of Al , 1 and 2ppm of Cd and 10, 15, 20, 25 and 30ppm of Zn. The culture flask were continuously shaken at 120rpms at 28℃ under illumination of 20 mol/m^2 · sec. After 4 days, the cells were centrifuged at 4℃ for 10minutes at 6,000rpm. The pellet was resuspended in 50mM Tris/HCl buffer (pH 7.5) twice, broken using liquid nitrogen and then centrifuged at 14,000rpm. The supernatant was used for protein analysis.

2) Quantification of protein

Bovine serum albumin was used as a standard according to Lowry *et al.* (1951). Absorbance determinations for protein determinations were performed at 650 nm in a spectrophotometer.

3) Electrophoresis

One dimensional electrophoresis was performed according to Laemmli(1970). Combine protein sample and 5×sample buffer(0.25mM Tris-HCl, 2% SDS, 20% glycerol, 10% mercaptoethanol, 0.004% bromophenol blue, pH 6.8). Heat at 100℃ for 2~10 minutes. After centrifugation, run a gel. Separating gel consists of 8% acrylamide, 0.375M Tris-HCl pH 8, 0.1% SDS, 0.0025% TEMED and 0.1% ammonium persulfate gel. Stacking gel consists of 3% acrylamide, 0.125M Tris-HCl pH 6.8, 0.1% SDS에 0.1% TEMED and 0.05% ammonium persulfate gel.

4) Gel staining

After electrophoresis, gel was stained at 0.05% R-250 Brilliant Commassie Blue, 50%

Table 1. r and K values of heavy metal treatments in Cyanobacterium *synechocystis* sp. PCC 6803

	Concentration(ppm)	r^a	$K^b(\times 10^5/ml)$
Control	0	0.03116	31.58
Al	5	0.03547	29.51
	10	0.03147	34.76
	15	0.03422	34.28
	20	0.03003	33.65
	25	0.02890	33.65
	30	0.02835	32.06
	35	0.14633	10.10
Cd	0.05	0.02604	33.26
	0.1	0.04683	27.44
	0.2	0.03303	25.85
	0.5	0.04067	25.69
	1	0.02974	15.03
	2	0.12353	10.89
Zn	5	0.03465	36.99
	10	0.02397	34.44
	15	0.03603	24.58
	20	0.02309	20.60
	25	0.05035	11.53

r^a : rate of population growth per capita

K^b : upper asymptote or maximal value of N

methanol, 10% acetic acid for 10 minutes and then was destained at 50% methanol, 10% acetic acid. After destaining, gel was stored at 10% methanol, 5% acetic acid.

RESULTS AND DISCUSSION

1. Growth changes by heavy metal treatment

1) Growth changes by Al treatment

When various concentrations of Al were added, growth change is expressed in Fig. 1. When exposed to Al from 5ppm to 30ppm, *synechocystis* grows normally. But more than that retard the growth of algae notably.

A coefficient of correlation between Al concentration and growth rate was negative value, -0.6710 ($p=0.049$) (Table 2). But, the results of t-test showed there was not siginificant difference between growth rate of control and that of experimetal groups (Table 3).

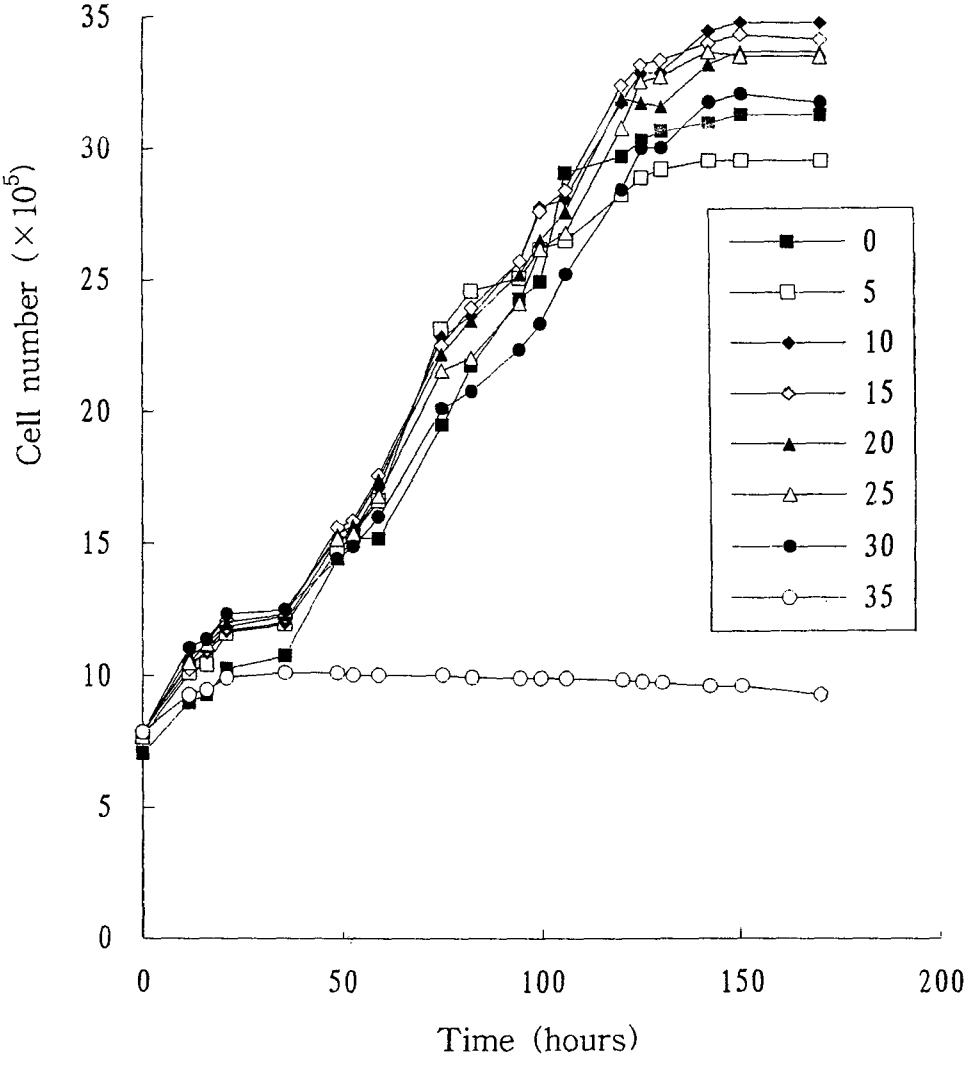

Fig. 1. The growth change of Cyanobacterium *synechocystis* sp. PCC 6803 by aluminum(Al) treatment.

Table 2. Correlation analysis of heavy metal concentration with r and K values in Cyanobacterium *synechocystis* sp. PCC 6803

	r	K
Al	−0.6710* P = .049	0.3310 P = .213
Cd	−0.7166 P = .055	−0.8863** P = .009
Zn	−0.0884 P = .434	−0.9409** P = .003

*P<0.05, **P<0.01

Table 3. Difference analysis of r and K values between Al treatment and control groups by t-test in Cyanobacterium *synechocystis* sp. PCC 6803.

Concentration	r		K	
(ppm)	t value	Probability	t value	Probability
5	−6.18	0.025*	2.32	0.147
10	−0.58	0.618	−2.04	0.178
15	−5.55	0.031*	−0.76	0.526
20	−0.10	0.927	−1.22	0.346
25	−0.55	0.640	−0.66	0.579
30	2.11	0.170	−0.26	0.818
35	−9.70	0.01**	88.50	0.000***

* $P<0.05$, ** $P<0.01$, *** $P<0.001$

A coefficient of correlation between Al concentration and maximal value of population size (K) was 0.3310 ($p=0.213$). That is to say, there are no correlation (Table 2). The resuls of t-test between Al concentrations and K showed that there was siginificant difference between K of control and K of 35ppm experimental group (Table 3).

In spite of the common mode of action of all these test metals, a very low toxic potential of Al was observed in short-term treatment which, however, reached the level of other metals(Cd, Cu, Ni, Fe). LC_{50} of Al was 12ppm and toxicity was lower than other metal in *Anabaena doliolum* under short-term treatment (Nirupama, 1994). This could be attributable to the slow uptake of Al as it prefers only "O" ligand domains of the cell membrane for binding compared to O, N and S for Cd and Fe, and N and S for Cu and Ni. Further, the very slow ligand exchange rate of Al as compared to other metals could be the other possible reason for its delayed toxicity (MacDonald, 1988).

2) Growth changes by Cd treatment

The results obtained are shown in Fig. 2 when Cd was given from 0.05ppm to 2ppm. There was no difference from control when exposed to 0.05ppm Cd. Maximal values of population exposed 0.1ppm and 0.2ppm Cd was 27.44×10^5/ml and 25.85×10^5/ml, respectively. Exposed to 0.5ppm Cd, K value has reduction similiar to 0.1 and 0.2ppm treatment group and population growth rate has no significant change. Under 1 and 2ppm Cd stress, growth was greatly diminished.

A coefficient of correlation between Cd concentratioin and growth rate was negative value, −0.7166 ($p=0.055$). But, the results of t-test showed there was no siginificance.

There was negative correlation, −0.8863 ($p=0.009$) between Cd concentration and K value (Table 2). As compared K of experimental group to that of contron by t-test, there was no siginificant difference in 0.05ppm and some inhibition in 0.1ppm and 0.2ppm ($p=0.021$, $p=0.013$, respectively). As concentrations were more than 0.5ppm, there are notable difference from control (Table 4). Exposed to 1ppm and 2ppm Cd, inhibition was greater

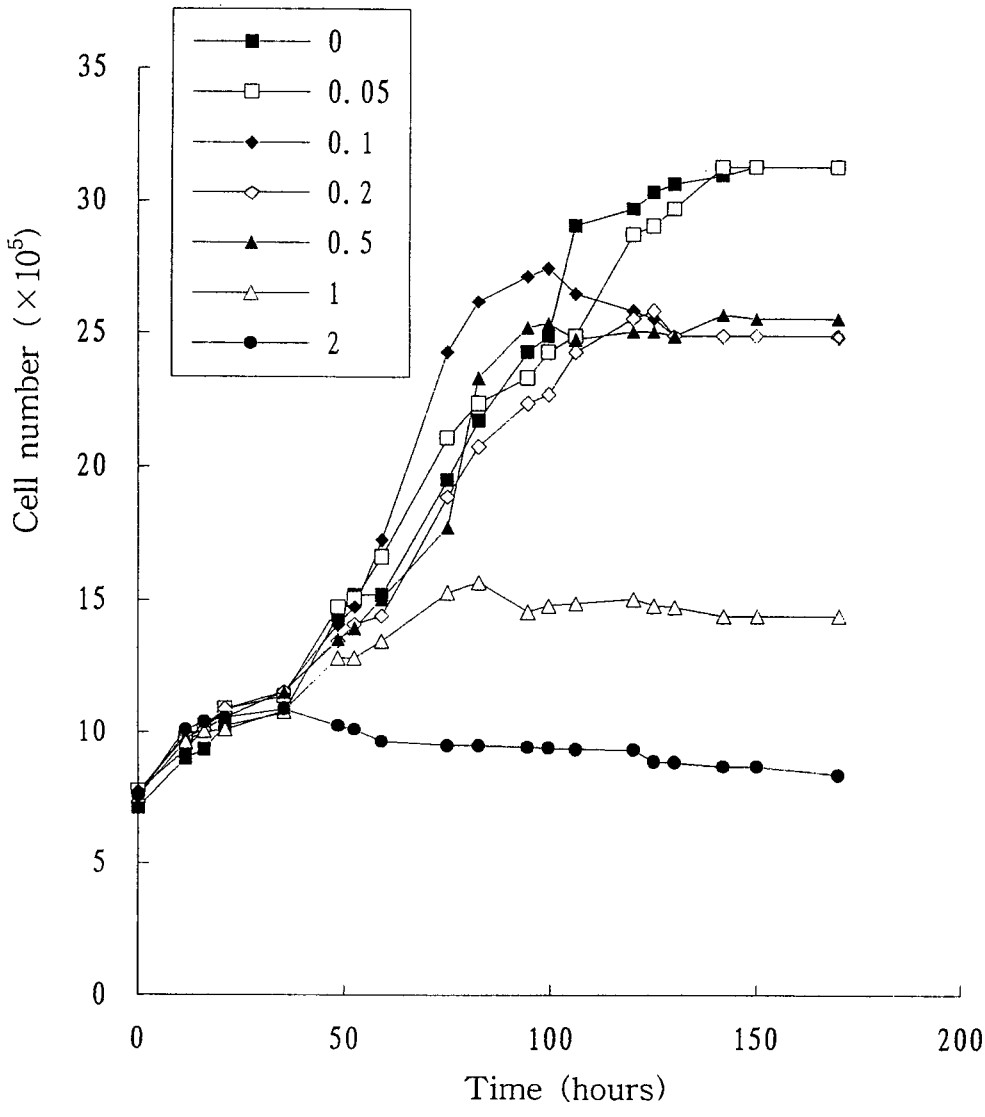

Fig. 2. The growth change of Cyanobacterium *synechocystis* sp. PCC 6803 by cadmium treatment.

and growth stopped.

Cyanbacterium *synechocystis* sp. PCC 6803 has higher resistance than Anabaena of which LC_{50} is 0.05ppm and lower resistance than green algae, *Chlorella regularis* which was not effected when exposed to 0~20ppm Cd (Sakaguchi *et al.*, 1979).

3) Growth changes by Zn treatment

The results of Zn treatment is given in Fig. 3. Zn had dual effect differently from other

Table 4. Difference analysis of r and K values between Cd treatment and control groups by t-test in Cyanobacterium *synechocystis* sp. PCC 6803

Concentration (ppm)	r		K	
	t value	Probability	t value	Probability
0.05	1.44	0.286	−0.72	0.544
0.1	−6.34	0.024*	6.86	0.021*
0.2	−2.61	0.121	8.61	0.013*
0.5	−2.48	0.002**	10.65	0.009**
1	0.55	0.636	35.85	0.001***
2	−8.90	0.012*	43.81	0.001***

* $P<0.05$, ** $P<0.01$, *** $P<0.001$

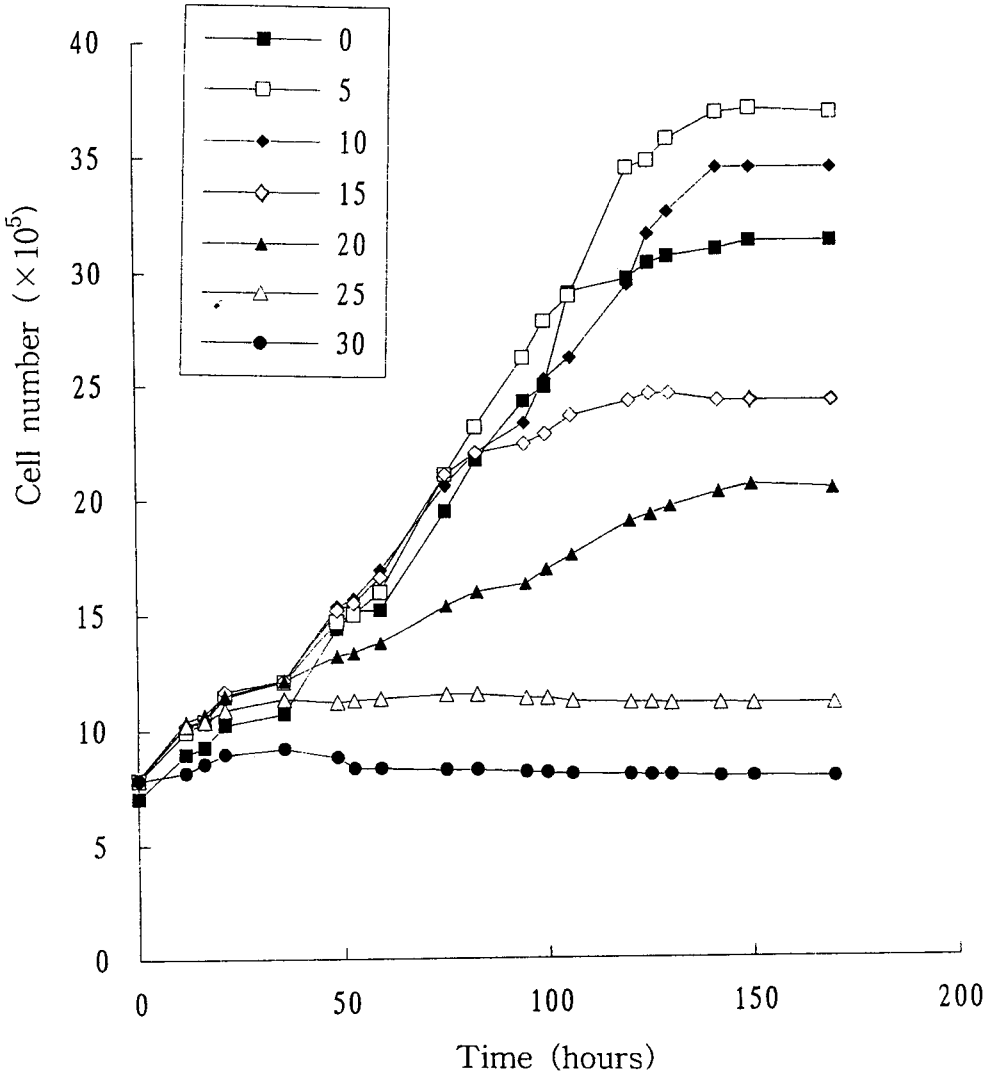

Fig. 3. The growth change of Cyanobacterium *synechocystis* sp. PCC 6803 by zinc treatment.

Table 5. Difference analysis of r and K values between Zn treatment and control groups by t-test in Cyanobacterium *synechocystis* sp. PCC 6803

Concentration	r		K	
(ppm)	t value	Probability	t value	Probability
5	−9.26	0.011*	−5.52	.031*
10	3.77	0.064	−3.82	.062
15	−8.27	0.014*	38.18	.001***
20	3.78	0.063	75.18	.000***
25	−3.59	0.070	28.21	.001***

* P<0.05, ** P<0.01, *** P<0.001

metals. The growth of algae in media containing 5ppm was stimulated. As concentration increases more than 15ppm, growth inbition increases. Under 25ppm Zn stress, growth was greatly diminished. There was no siginificant change of population growth rate (r) under Zn treatment similiary to other metals.

Correlation coefficient between Zn concentration and r value was −0.0884. So, there was no siginificant correlation under treatment concentration. But, correlation coefficient between Zn concentration and K value was −0.9409 ($p=0.003$), negative value. t-test of this result showed there was meaningful growth facilitation exposed to 5ppm Zn ($p=0.031$) and 10ppm Zn treatment was similar to control. But, Zn more than 15ppm decreased K value as concentration increases(Table 5).

In some environments, organisms exist near the asymptotic density(K) for much of the year, and these organisms are subject to K selection. In other habitats, the same organisms may rarely approach the asymptotic density but remain on the rising sector of the curve for most of the year; these organisms are subjected to r selection. MacArthur and Wilson(1967) defined r and K selection to be density-dependent natural selection. As a polulation initially colonized an empty habitat, r selection would predominate for a time and ultimately the population would come under K selection.

According to logistic theory, r and K values of each heavy metal-treated groups were estimated. Correlation analysis of r and K values with metal concentration showes that there is negative correlation between K and concentration in Cd and Zn treatments. Critical concentration which shows lethal or sublethal effect was estimated by t-test of each r and K value.

When Al, Cd and Zn were added to Cyanobacterium *synechocystis* sp. PCC 6803, r value didn't changes and K value decreased as metal concentration increased. Cyanobacterium *synechocystis* sp. PCC 6803's selection mechanism against heavy metal was K selection. As metal concentration increases, cell stopped population growth and responsed to use their resource more efficiently.

2. Protein synthesis change by heavy metal treatment

The cells cultured in 10, 20, 30, 40 and 50ppm of Al, 1 and 2ppm of Cd, and 10, 15, 20, 25

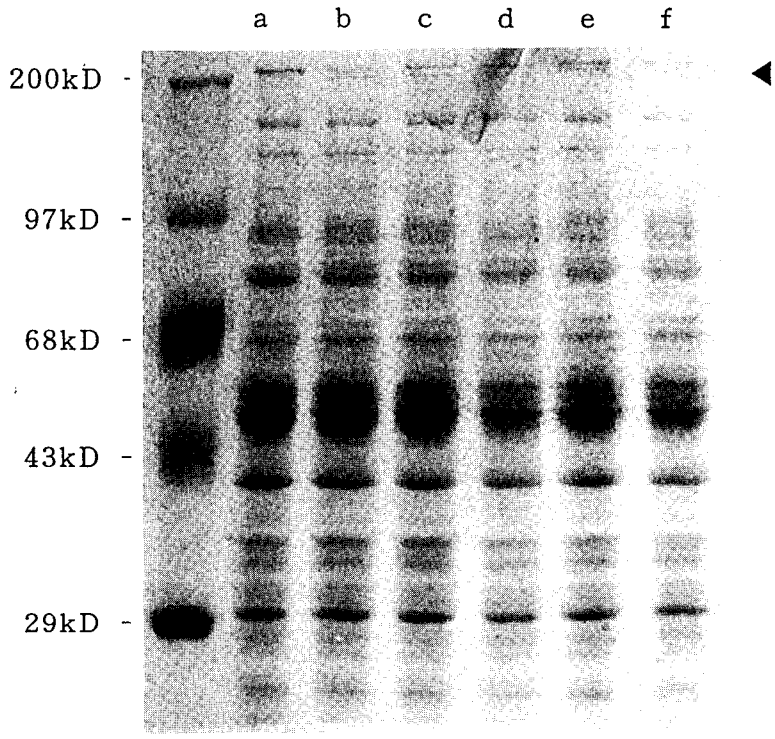

Fig. 4. SDS-PAGE of crude extract of aluminum treatment and control groups in Cyanobacterium *synechocystis* sp. PCC 6803.
a. Control b. Al 10ppm c. Al 20ppm d. Al 30ppm
e. Al 40ppm f. Al 50ppm

and 30ppm of Zn for 4 days was used for protein analysis. Analysis of protein synthesis with SDS-PAGE showed alterations of protein synthesis pattern.

Culture for protein analysis didn't show growth inhibition equal to test-tube culture for growth analysis. Continous shaking provided nutrient and larger space, so effect of metal decreased. This correspond to the report that higher nutrient reduces toxicity heavy of metals다(Mallick, 1994).

Using Lowry method, extracted protein was quantified. And same amount of protein was analyzed by electrophoreis. The synthesis of protein about 220kD increased markedly.

The effect of heavy metal on organism is that metal changes many biochemical reaction in organism. Metal causes change of cell membrane and effects on permeability. In most case, metal binds active site of enzyme such as SH group and inhibits enzyme activity in cell. Also, metal binding nonactive site of enzyme changes electric current of active site or protein conformation to inhibit activity. It affects metal metabolism necessary to organism and enzyme activity through substitution reaction. But, all metal doesn't bind im-

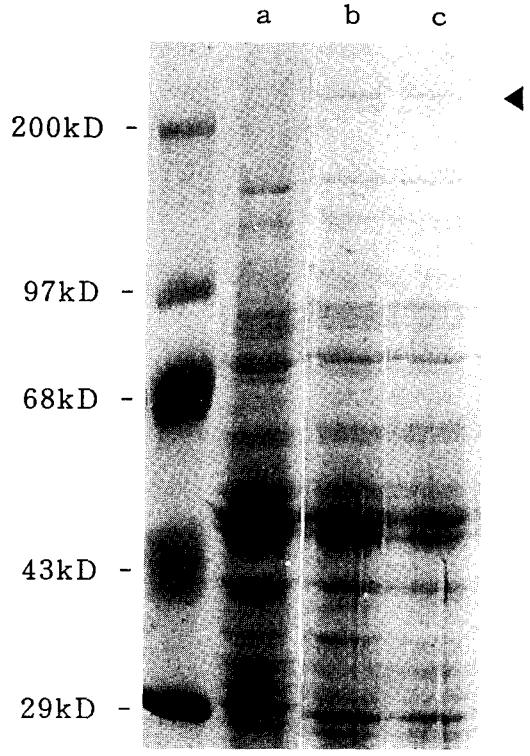

Fig. 5. SDS-PAGE of crude extract of cadmium treatment and control groups in Cyanobacterium *synechocystis* sp. PCC 6803.
a. Control b. Cd 1ppm c. Cd 2ppm

portant enzyme in cell. It binds various protein not to effect on any metabolism in organism. Metallothionein is repesentative protein binding heavy metal to reduce its toxicity. Until now, this protein has been studied mostly in animal such as rat, human, cat fish bluegills, xenopus, mouse, polychaete.

When Zn and Cu were added to Polychaete with sublethal level, large metalloprotein more than 60kD and 20kD metallothionein having higher affinity for Zn were induced. (Macano, 1996). Metal binding protein was detected in microbes such as yeast and *E. coli* and higher plants such as tomato, cabbage and beans.

But, the study about macromolecule level in algae has been relatively insufficient. In case of Cyanobacterium *synechocystis* sp. PCC 6803, various concentration of salts inhibits growth, changes enzyme activity and induces macromolecule synthesis change to adapt to environmental changes. Especially, protein undergoes quantitative and qualitative change (Hageman *et al.*, 1994).

Until now, size of most protein induced by metal was less than 60kD. As larger protein induced in this study was different from other study, it is necessary to research this protein in detail.

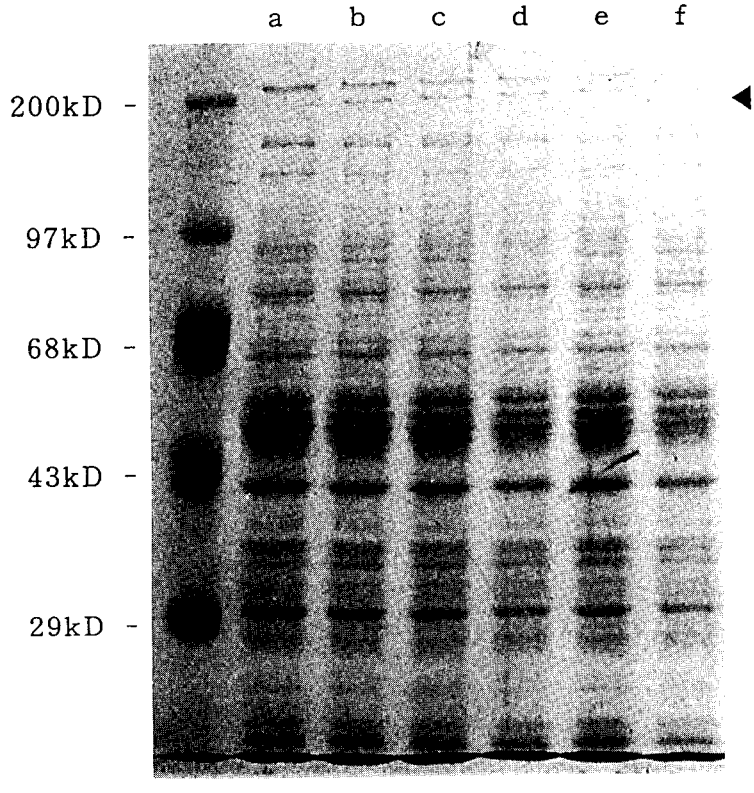

Fig. 6. SDS-PAGE of crude extract of zinc treatment and control groups in Cyanobacterium *synechocystis* sp. PCC 6803.
a. Control b. Zn 10ppm c. Zn 15ppm d. Zn 20ppm
e. Zn 25ppm f. Zn 30ppm

적 요

Cyanobacterium *synechocystis* sp. PCC 6803에 aluminum (Al), cadmium (Cd), zinc (Zn)을 처리하여 생장 변화와 단백질 합성 유형의 변화를 살펴보았다.

Al의 경우 5ppm에서 30ppm까지는 정상적인 생장을 보였으나 그 이상을 처리하면 치명적인 억제현상이 나타났고, Cd는 0.05ppm에서는 생장에 영향을 미치지 않았으나 0.1, 0.2, 0.5ppm에서는 약간의 생장 억제가, 1ppm과 2ppm에서는 심각한 생장억제현상을 나타냈다. Zn의 처리 시에는 이중효과가 나타났다. 5ppm의 Zn을 처리하면 생장 촉진이, 15ppm이상의 Zn을 처리하면 농도가 증가하면서 생장 억제가 커지다가 25ppm이상에서는 거의 생장을 하지 않았다. 로지스틱 이론을 이용하여 각 중금속 처리군의 K값과 r값을 구하여 상관관계와 t-test 분석 결과, Cd와 Zn에 대한 Cyanobacterium *synechocystis* sp. PCC 6803의 저항기작은 K 선택임을 알 수 있었다.

15ppm과 25ppm의 Al 및 Zn, 1ppm과 2ppm의 Cd를 처리한 후 SDS-PAGE를 통해 단백질을 분석해 본 결과 약 220kD의 단백질 합성이 현저하게 증가하였다.

본 연구에서는 Al, Cd와 Zn에 대한 Cyanobacterium *synechocystis* sp. PCC 6803의 저항기작이 *K* 선택임을 밝혔고, 중금속 처리에 의하여 단백질 합성 유형이 변화되었음을 규명하였다.

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