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Studies on Cd and Zn Removal Ability and Detoxification of *Oenanthe stolonifera*

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미나리(*Oenanthe stolonifera*)의 Cd, Zn 제거능과 내성에 관한 연구

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ABSTRACT

To examine the possibility of biomonitoring of heavy metal pollution in water and soil, a study was performed to investigate the heavy metal removal ability and metal-binding protein (MBP) as detoxification process using *Oenanthe stolonifera*. After *O. stolonifera* was exposed to individuals (cadmium, zinc) and mixture (cadmium+zinc) for 4 days, removal rate of heavy metal and pH in the treatment medium was measured. MBP was assayed by means of ion exchange column chromatography. The exposure to mixture (Cd:76.8%, Zn:75%) rather than individuals (Cd:82.9 %, Zn:90.4%) showed a synergism raising the toxic effect. Initial removal rate was different for each heavy metal: in case of exposure to cadmium it was over 60% on day 1, while for zinc it was 75~90% on day 4. Throughout the experimental period, pH value of treatment medium continuously decreased, since cortex in the roots may secret organic acid to adjust and prevent toxicity of metals. The existence of MBP in the 70~80 fraction and the presence of Zn-enzyme pool was ascertained with the column chromatography. This study demonstrated a possibility that heavy metal removal ability of *O. stolonifera* resulted from detoxification process and MBP could be utilized as a biomarker of heavy metal pollution.

Key words: Cadmium, Detoxification, Metal-binding protein, *Oenanthe stolonifera*, Zinc

INTRODUCTION

Soil and water has been polluted with heavy metal and sewage released as byproducts of industrial process or discharged from rested and abandoned mines (Cho and Kim 1995). Heavy metal acts as a stress factor upon plant which decreases its activity and growth. The plant's response to heavy metal can be shown in two phases: sensitivity and resistance. Sensitivity is a symptom related to the damage effect of stress on the plant. Since heavy metal is divalent cation (Ca^{2+}, Zn^{2+}, Pb^{2+}, Cu^{2+}) which is replaced by the cation of plant inside, toxicity is enhanced due to disequilibrium in ion distribution (Blake *et al.* 1987). Resistance indicates how plant grows and reproduces well in spite of chronic stress of heavy metal (Cho and Kim 1995). It is generally referred that *Zizania caduciflora* (wild rice), *Typha orientalis* (Cho and Kim 1995), *Spirodela polyrhiza* (duckweed) (Oh and Park 1986), water hyacinth (Lenka *et al.* 1990), *Oenanthe stolonifera* (parsley) (Kwon and Na 1994), and redshrank (Lee and Yun 1994) are aquatic plants that tended to resist heavy metal. Living things have evolved the defense with detoxification making it possible to sustain the normal physiological mechanism. For example metal binding protein (MBP) or metallothionein (MT) was induced in the livers of human and rat. MT plays a role in storing up essential elements as well as heavy metals and is the protein which can relieve toxicity in the system exposed to an excessive quantity of heavy metals. It is documented that Cd-binding protein can be induced in water hyacinth, an aquatic plant with the removal ability of heavy metals (Fujita and Kawanishi 1986), and it is already used as an FAMS(Floating Aquatic Macrophyte based treatment System) model and utilized in biomonitoring of polluted water in the countries (Reddy and Tucher 1983, Orth and Sapkta 1988). FAMS model, unlike chemical systems, can purify water without causing damage by second products. In Korea, researches with wild rice, duckweed, water hyacinth and parsley applied to the system for purification treatment are in progress at laboratory level.

The purpose of our study is to confirm the feasibility of utilizing metal-binding protein as a biomarker and purifying sewage by means of *O. stolonifera*. Moreover it can be used to recover the ecosystem and to economically purify heavy metal pollution in the mine area by using the plants as a source of methane gas which is produced by anaerobic fermentation.

MATERIALS AND METHODS

Plant materials

O. stolonifera seeds were obtained from the Department of Agriculture of Seoul National University. After germination, young plants were cultivated until their roots grew to 10 cm and transplanted to 24 porcelain pots (diameter; 14 cm, height; 12 cm) with 15 individuals per pot cultured in condition of 4000 lux, day 16 hours night 8 hours, and 25± 1℃ for 4 weeks in growth chamber (Percival, Model E-54u).

Measuring removal ability of Cd and Zn

In order to expose *O. stolonifera* to Cd and Zn, 0, 0.5, 1, 1.5 ppm of $CdCl_2$ and 0, 1, 3, 9 ppm of $ZnSO_4$ were added to 1*l* of the medium, respectively. To expose to mixture of Cd and Zn, 1 ppm of $CdCl_2$ and 3 ppm of $ZnSO_4$ were added.

The entire period of the experiment consists of 4 days of exposures. To investigate heavy metal elimination tendency of *O. stolonifera*, Cd and Zn concentration was measured three times repeatedly after 4 days and every 24 hours. In order to analyse the concentration of heavy metals, nitric acid-hydrochloric acid digestion (Joung 1994) followed by an atomic absorption spectrophotometer was used (Perkin-Elmer, 2380).

Investigation of detoxification

1) pH measurement

pH value of the treatment medium during the experimental period was measured 3 times repeatedly for each trial everyday (2:00 pm) by pH meter (Metter Toledo, chekmate system).

2) Assay for metal-biding protein (MBP)

After treating with 1 + 3 ppm of Cd+Zn, 1 ppm of Cd ($CdCl_2$), 3 ppm Zn ($ZnSO_4$) in the experimental pots, *O. stolonifera* was cultivated for two weeks. Roots for assay were sampled and washed several times by deionized water. MBP was extracted by Fujita and Kawanish (1986) method.

RESULTS AND DISCUSSION

Heavy metal removal ability

To examine Cd and Zn elimination ability of *O. stolonifera* when it was treated individually and by mixture, heavy metal concentation of nutrition solution was measured after 5 days of cultivation period (Table 1). It was found that natural removal rate of heavy metal caused by vaporization and uptake of inside surface in the pots were 4% in the case of exposure to Cd, 8% in case of Zn, and 6.8% (Cd), 6.6% (Zn) in case of Cd+Zn. Considering this result, removal rate of heavy metal was more than 70% in the case of individual exposure either to Zn or Cd. Also in comparison with individual treatment Cd 1 ppm and Zn 3 ppm and mixture treatments shows a tendency of reduced removal rate. This suggests that when exposed to mixture of two kinds of heavy metals, removal rate of heavy metals in *O. stolonifera* is decreased as a result of synergism of enhancing the toxic effect. *O. stolonifera* could be applied to actual purification system of water or soil as seen by the result of more than 70% removal of heavy metals.

In order to study the time variation of removal rate of heavy metal in the treatment medium, it was measured and compared every 24 hours for 4 days (Fig. 1, Fig. 2). When the plant exposed to Cd, Zn individually and by mixture, the removal ability was observed to be different from each other. In the case of Cd, after 1 day treatment, a high initial removal rate of 60.6~65.2% was measured at experimental concentration, and then through 4 days increased gradually. That is to say, remainder Cd concentration of the treatment

Table 1. Removal rate of Cadmium (Cd) and Zinc (Zn) in the medium treated with Cd, Zn and Cd+Zn for five days

Treatment(ppm)	Removal rate(%) ± SD	Treatment(ppm)	Removal rate(%) ± SD
Cd 0.5	89.2 ± 9.78	Zn 1.0	75.2 ± 3.05
Cd 1.0	82.9 ± 11.69	Zn 3.0	90.4 ± 1.50
Cd 1.5	84.1 ± 2.20	Zn 9.0	81.5 ± 5.33
Cd+Zn(1+3)	76.8 ± 4.13	Cd+Zn(1+3)	75.0 ± 13.70

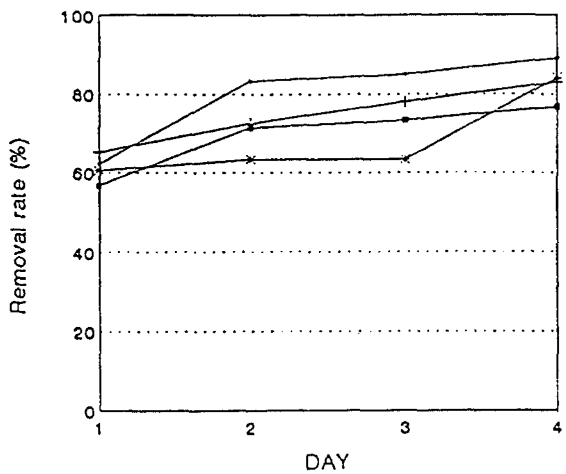

Fig. 1. Daily removal rate (%) of Cd in the medium treated with Cd and Cd+Zn. Cd 0.5ppm (−□−), Cd 1ppm (−|−), Cd 1.5ppm (−✳−), Cd+Zn 1+3ppm (−■−).

medium was checked, which has a decreasing removal ability slowly from the second day (Fig. 1). High initial removal rate of Cd was due to the removal by the root's absorption (Kwon and Na 1995). It is thought that slowly increasing tendency of removal rate from the second day was a result of detoxification response of *O. stolonifera*. In the case of Zn, initial removal rate was as low as 21.75~28.16%. In contrast to Cd, the removal rate tended to increase to 75.25~90.41% from the second day to the forth day for Zn (Fig. 2). Also in the case of mixture of Cd+Zn, initial removal rates of Cd and Zn were 56.75% and 26.66%, respectively.

This seems to be due to the fact that replacement rate tendency of Cd (10^8/s) was higher than that of Zn (10^7/s) (Joung 1994). This point needs further study. The result suggests that if Zn is lacking in a plant, the removal rate of Cd will be further increased (Bayne *et al.* 1985).

Heavy metal detoxification process

1) pH value change in the medium

pH value of the treatment medium measured for 4 days had an acidifying tendency. The range was from weak acid (5.58±0.05) to strong acid (4.50±0.34) (Fig. 3, Fig. 4). Despite this acidity, there was no significant difference between the growth rates of *O. stolonifera* in the treatments and in the control. So it seems that the optimum pH for the growth of the plants is actually closer to acid than neutral. It was suggested that, as a part of heavy metal detoxification process, plants secrete organic acid from the cortex in the roots to

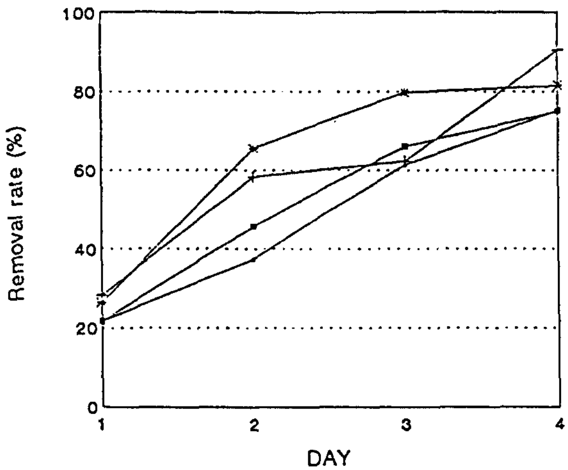

Fig. 2. Daily removal rate (%) of Zn in the medium treated with Zn and Cd+Zn. Zn 1ppm (−□−), Zn 3ppm (−|−), Zn 9ppm (−✱−), Cd+Zn 1+3ppm (−■−).

prevent metal toxicity (Kinshinami and Widholm 1987). By the way, it was demonstrated, that the pH value varied: 4.53±0.16 when treated with Cd and 4.15±0.05 when treated with Zn. It was observed that the acidifying tendency was stronger when treated with Zn than treated with Cd. These findings indicate that *O. stolonifera* is a Cd and Zn-resistant species. Nishizone asserts that organic acids are secreted in the order of citrate ≫ malate > succinate > fumarate from the wild type of *N. plumbaginifolia*, and that they are secreted by Cu and Zn-resistant species. In case of exposure to mixture of Cd and Zn, a relatively slow reduction was observed in the pH curve. This could be originated from the reduced secretion of organic acid caused by a higher toxic effect in mixture treatment than in individual treatment.

2) Metal-binding protein assay

It was reported that there are two pathways of living things to reduce the toxic effect of heavy metal (Brown *et al.* 1980). One is to bind heavy metals with metallothionein (MT) or accumulate them in the lysosome. The other is through pinocytosis or membrane-bound compartmentation. For instance vertebrates (human, rabbit, rat) induce metallothionein that can reduce toxic effect by means of binding heavy metals with protein in their liver. However, in invertebrates (mussel, oyster, crab, earthworm) each species has different inducer organs of MT (Dallinger and Rainbow 1992). Some surveys showed the

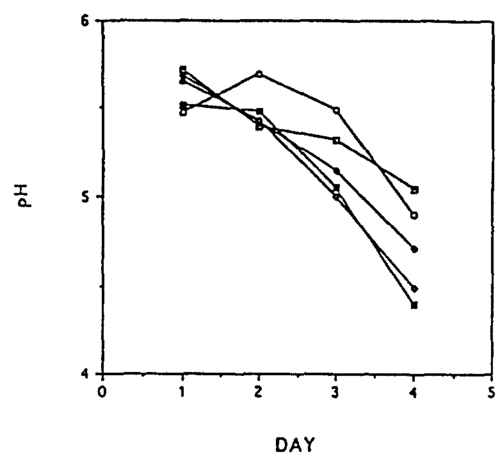

Fig. 3. Daily variation of pH in the medium treated with Cd and Cd+Zn. Conytrol (−□−), Cd 0.5ppm (−◆−), Cd 1.0ppm (−■−), Cd 1.5ppm (−◇−), Cd+Zn 1+3ppm (−○−).

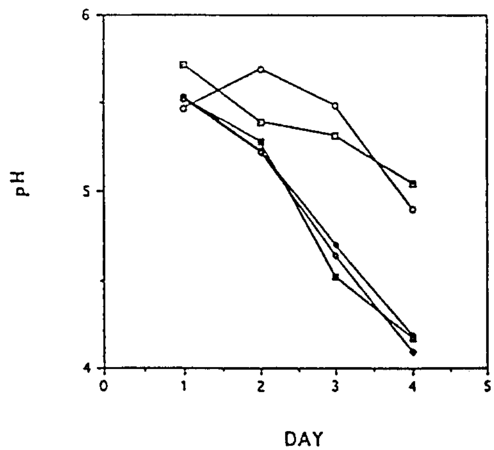

Fig. 4. Daily variation of pH in the medium treated with·Zn and Cd+Zn. Control (−□−), Zn 1ppm (−◆−), Zn 3ppm (−■−), Zn 9ppm (−◇−), Cd+Zn 1+3ppm (−○−).

fact that metal-binding proteins (MBP) of plant have little different from metallothionein of animals (Bartolf *et al.* 1980, Bayne *et al.* 1985, Berger *et al.* 1995). MBP of aquatic plants has a much lower molecular weight of 4,000 Da than metallothionein and consists of 2 cadystin A [(r-Glu-Cys)3-Gly] and 2 cadystin B[(r-Glu-Cys)2-Gly] in 4 subunits (Fujita and Kwanish 1986). We took measurements by ion exchange column chromatography to confirm whether or not the root of *O. stolonifera* induce MBP when exposed to Cd and Zn (Fig. 5). Fig. 5 describes the amount of protein and heavy metals natively. Fig. 6 showed that there is a small amount of MBP in unpolluted environment, but when exposed to heavy metals, a large amount was induced. MBP was induced through 70~80 fractions in high amount of heavy metal, and in 40~80 fractions only high amount of Zn was observed indicating the presence of a Zn-enzyme pool.

From the results of removal rate measurments, we think that for individual exposure to Zn, Zn is contained in a Zn-enzyme pool and the remainder is stored by binding with MBP and is used when there is a shortage of essential element. If Zn is lacking in the Zn-enzyme pool of a plant, the removal of Cd will highly increase in a Cd-contaminated environment than in an unpolluted environment.

Thr results of the study showed that there are two differences from those of other higher plants (Bartolf *et al.* 1980). First is that in 0~30 fractions in the treated plant, heavy metal level was measured to be low in contrast with that of protein which was not shown in the control. This finding suggests that an unknown new protein other than MBP was induced in exposure to heavy metal. Second, it was demonstrated that in exposure to mixture MBP exists in 65~70 fractions and 75~80 fractions and a high peak of heavy metal occurs in 65~70 fractions for Cd, and in 75~80 fractions for Zn (Fig. 7). It has not yet been reported whether MBP induced by heavy metals varies with the heavy metals or is only one kind, but it is suggested that different kinds of MBP is of *O. stolonifera* bind each Cd and Zn.

The study by Davin *et al.* (1984) showed the same result that the different MBPs for Cd and Zn were also induced in the root of rice.

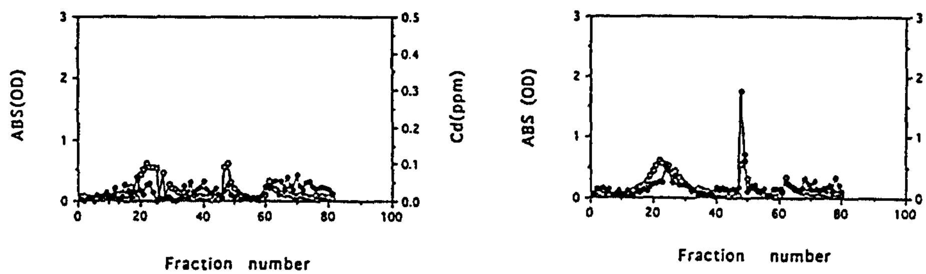

Fig. 5. DEAE-cellulose column chromatography of the tissue extract from roots of *Oenanthe stolonifera* cultivated in mineral medium as control. After the column washed with buffer without KCl, the adsorptives were elluted stepwise with 0.2 and 0.4 M KCl. ○, A_{280}; ◆, Cd and Zn in ppm.

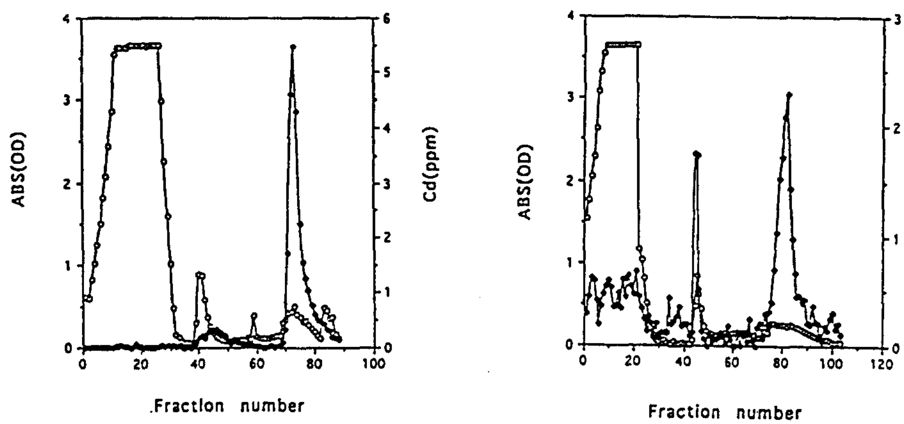

Fig. 6. DEAE-cellulose column chromatography of the tissue extract from roots of *Oenanthe stolonifera* cultivated in mineral medium treated with Cd and Zn. After the column washed with buffer without KCl, the adsorptives were elluted stepwise with 0.2 and 0.4 M KCl. ○, A_{280}; ◆, Cd and Zn in ppm.

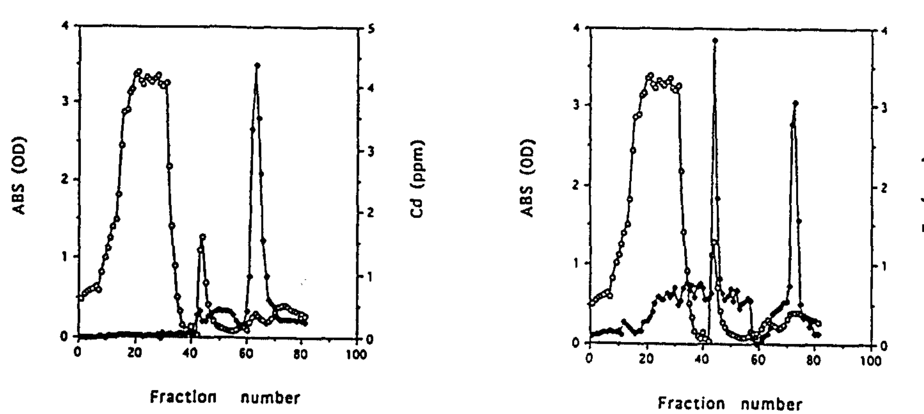

Fig. 7. DEAE-cellulose column chromatography of the tissue extract from roots of *Oenanthe stolonifera* cultivated in mineral medium treated with Cd+Zn. After the column washed with buffer without KCl, the adsorptives were elluted stepwise with 0.2 and 0.4 M KCl. ○, A_{280}; ◆, Cd and Zn in ppm.

적 요

수질 정화능이 뛰어난 정수 식물로서 국내에서 자생하고 식용으로 널리 재배되는 미나리를 이용하여 수질 및 토양내의 중금속 오염정도를 biomonitoring할 수 있는지에 중점을 두어 중금속 제거능 및 내성기작의 일부인 금속결합단백질(MBP)에 대해 조사하였다. 중금속 제거능은 4일 동안 단일 처리 (Cd, Zn)와 혼합 처리 (Cd+Zn) 하여 제거율을 비교하였으며, 제거 경향도 조사하였다. Cd의 경우는 단일 처리시 (82.9%) 가 혼합 처리시 (76.8%) 보다 제거율이 높게 나왔고, Zn도 90.4%, 75%로 단일 처리시 높게 나타났다. 이것은 혼합 처리가 단일 처리시보다 더 독성효과가 커지는 상승작용에 기인하여 제거율이 떨어지는 것으로 사료된다. 중금속의 종류에 따라 초기 제거율이 다른데 Cd은 처리 1일째에서 60% 이상이었고, Zn은 처리 4일째에 75~90%의 제거율을 보였다. 중금속 내성을 보이는 동안 처리조 배양액의 pH 변화 및 뿌리의 MBP를 조사한 결과, 실험기간 동안 중금속 처리구는 대조구와 비교해서 배양액을 산성화했는데, 이는 중금속 노출시 뿌리에서 피층 세포로부터 유기산을 분비하여 독성으로부터 보호하고 적응하는 내성작용의 일부인 것으로 생각된다. 중금속 독성에 대한 근본적인 내성을 나타낼 수 있게 한 MBP 유도를 column chromatography를 통해 70부근과 80부근의 분획에서 확인하였고, Zn-enzyme pool은 40~50 분획에서 존재함을 알 수 있었다.

본 연구는 미나리의 중금속 제거능이 미나리의 내성에 의한 결과임을 밝혔고, 수질의 중금속 오염 biomarker로서 MBP를 이용할 수 있으리라는 가능성을 제시하고 있다.

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