Korean J. Ecol. 28(1): 7~13, 2005

Phytoremediation of Cu-contaminated Soil and Water by *Commelina communis*

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ABSTRACT: In the present study, we investigated the tolerance of *Commelina communis* to growth in Cu-contaminated soil and water. We examined the germination rate, root and shoot growth of seedlings, fresh biomass in soil and water, and ability to eliminate Cu. We found that *C. communis* eliminated 41% of Cu in soil containing 50 mg Cu/kg and removed over 50% of Cu from water containing 100 mg Cu/L Cu. In addition, the plants could accumulate 90 mg Cu/g when grown in soil containing 50 mg Cu/kg and 140 mg Cu/g when grown in soil containing 100 mg Cu/kg; thus higher levels of Cu removal were observed in soils containing higher Cu concentrations. In water, the maximal accumulation rate was 4.9 mg Cu/g root and 1.2 mg Cu/g shoot in water containing 20 mg Cu/L, and 7 days after exposure, Cu absorption saturated. Further, the growth rate of *C. communis* was not affected by up to 100 mg Cu/kg in the soil. Therefore, the phytotoxic effect of Cu on plants increased as the concentration of Cu was raised, although to different extents depending on whether the Cu was in soil or water. Overall, Cu removal from soil by *C. communis* was most effective at 100 mg Cu/kg in soil and 10 mg Cu/L in water. Finally, we identified two peaks of Cu-binding ligands in *C. communis*. Which is a high molecular weight peak (HMWL) at 60 kDa (Fraction 17 to 25) and a Cu binding peptide peak at <1 kDa (Very low molecular weight ligand: VLMWL). Cu-binding peptide (Cu-BP) was observed to have an amino acid composition typical of phytochelations.

Key words: *Commelina communis*, Copper, Phytoremediation

INTRODUCTION

Copper is an essential nutrient required by plants in small amounts as a chelate, but the presence of large amounts in the environment, especially in soil, can be dangerous. Total concentrations of Cu in normal soils are typically 2 to 250 mg/kg (Alloway 1995), and in drinking water, 2 or 1.3 mg/L according to the World Health Organization (WHO) and the United States Environmental Protection Agency (U.S. EPA, 2002), respectively. Cu is strongly bound to organic matter in the soil, which allows it to be mobile (Mengel and Kirby 1987). Major sources of Cu pollution include release from fertilizers and fungicides, and sewage sludge (Levi-Minzi and Riffaldi 1978). Excess Cu and other metals can inhibit growth and photosynthetic activity in plants and may promote aging of plants (Wilmer and Stomata 1983). Accumulation of Cu in the human body can cause gastroenteric problems, depression, and kidney and liver disease. Therefore, it is important to remediate heavy metal-contaminated soils. Current methods of removing heavy metals include physical separation, acid leaching, and electrochemical processes. However these methods can be expensive, ineffective, and create secondary contaminated materials. For these reasons, we have explored phytoremediation as a method for eliminating heavy metals from soil. Phytoremediation is an inexpensive and ecological technology that uses plants to restore contaminated soil (Beak *et al.* 1999). In these plants, solar energy is utilized to convert the free, toxic metals into stable nontoxic complexes (Suthersan 1997).

Although Cu uptake by most plants is low, with plant Cu concentrations typically ranging from 2 to 20 ppm (Wallnofer and Engelhardt 1984), the growth of plants called hyperaccumulators is not affected by Cu, and these plants can remove high levels of Cu from soil (Antonovics *et al.* 1971). Plants normally have mechanisms for tolerance or resistance to Cu that are activated when they are grown in Cu-contaminated soil (Ernst *et al.* 1992). There have been many studies on Cu uptake and accumulation by plants. For example, Harper *et al.* (1997) tested yellow monkey flower (*Mimulus guttatus*) for Cu tolerance and Jackson *et al.* (1990) investigated the Cu resistance of *Silene cucubalus*. Moreover, the hyperaccumulator *Ipomoea alpina* has been shown to accumulate as much 12,300 mg/kg in its shoots (Baker and Walker 1990). Recently, many researchers have studied the use of phytoremediation. Neumann and Zur Nieden (2000) reported that *Cardaminopsis halleri* accumulates Zn and Cu, and Monni *et al.* (2000) showed that *Empetrum nigrum* absorbed high concentrations of Cu and Ni.

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The germination rate is usually used to search for resistant plants because it is very sensitive to the environment (Wang 1991). In particular, the sprouting period of germination is especially sensitive to the environment, and thus, the root and shoot lengths are often used as a standard measure of plant tolerance (Turner *et al*. 1991). Germination has, in fact, been used for selection of hyperaccumulator plants (Mohan and Hosetti 1991). A germination test for the selection of hyperaccumulator plants for phytoremediation at a contaminated site has been described (EPA 2001). However, there have been few long-term studies on the effects of hyperaccumulator plants on soil contamination.

The present studies were performed to investigate the use of *C. communis* in phytoremediation of Cu-contaminated soil and water. The integration of specially selected metal-accumulating plants with innovative soil amendment characteristics allows plants to achieve high biomass and metal accumulation rates from soils and water. One crop plant that produces high rates of biomass under field conditions is *Brassica juncea* (India mustard), which has been used successfully to decrease the selenium content of soils in California (Bańuelos *et al*. 1993). *C. communis*, a herbaceous plant indigenous to Korea, is highly resistant to a wide rage of heavy metal concentrations and has high rates of biomass growth. We examined the effect of the Cu concentration in soil and water on the germination rate, root and shoot growth, and fresh biomass of *C. communis* seedlings. To determine the ability of *C. communis* to absorb Cu, we studied the effect of the Cu concentration on the rate of Cu absorption.

MATERIALS AND METHODS

Phytotoxicity tests

Medicago sativa, *Abutilon avicennae*, *Commelina communis*, *Echinochloa frumentacea*, *Zea mays*, and *Helianthus annuus* were used in the present work. Seed germination and root elongation toxicity tests were performed (David *et al*. 1995). For Cu toxicity tests, each soil sample was artificially contaminated with 0, 50, 100, 200, or 300 mg Cu/kg of soil. Test pots containing 150 g of contaminated soil were planted with 10 seeds each and then placed in darkness at 25±2℃ for 14 days. At the end of the test period, if a shoot was visible, seeds were scored as germinated, shoot and root lengths were measured to the nearest centimeter, and biomass was determined. All tests were performed in triplicate.

Hydroponic experiment

Seeds from *C. communis* were sown in commercial potting mixture (B.P. #2, Hungnong Seed Company, Korea) and grown for 6 weeks in pots. Seedlings of similar height and fresh weight were selected and transplanted to aquatic bioreactors for hydroponic culture and were exposed to 4 L Hoagland solution containing 10 mg of Cu for 7 days.

Pot experiment

Sandy loam soil (5.6% organic matter; pH 8.6; consisting of 5.7% clay, 13.7% silt, and 80.6% sand) was collected from an agricultural site at Seoul, Korea. The soil was passed air-dried, through a 0.4-cm sieve, and then artificially contaminated with 50 mg of $CuCl_2$ per kilogram of soil. After the contaminated soil had been aged for 1 week, 1 kg of the soil was placed into each pot and 10 seeds were sown. All plants were grown for 30 days in a room maintained at 25℃ with 16 h light/8 h dark cycles. Uncontaminated soil was used as a control.

Analysis of Cu

The concentrations of total, exchangeable, and soluble Cu in the soil samples were measured. Soil samples were dried at room temperature and analyzed for water-soluble Cu by equilibrating 1 g of soil with 20 ml of 0.01 M KNO_3 for 2 h. The amount of exchangeable Cu in soil was estimated by extracting 1 g of the soil with 20 ml of 1 N NH_4COOH for 1 h. For the total Cu in soil, 0.5 g of soil was extracted with 2.4 ml of aqua regia (35% HCl 1.8 ml + 65% HNO_3 0.6 ml) in an MDS-2000 automatic micro-wave digester (CEM). The extracted Cu of each soil sample was analyzed by graphite and flame atomic absorption spectrophotometry (AAS analysis 100, Perkin Elmer), which was calibrated using certified reference materials (MESS-2 Marine Sediment; National Research Council of Canada).

Plants were harvested by gently removing them from the soil. Prior to analysis, plants were washed with water to remove soil deposits. To determine the amount of Cu in the plants, roots and shoots were further separated with scissors and then dried in an oven at 70℃ for 24 h. Plants samples were digested in conc. HNO_3 in an MDS-2000 automatic micro-wave digester (CEM). Cu contents were then determined with an AAS analysis 100 graphite-furnace atomic absorption spectrometer (AAS analysis 100, Perkin Elmer), which was calibrated using certified reference materials (No. 10-c Rice Flour-Unpolished; National Institute for Environmental Studies in Japan).

Purification of Cu-binding peptide (Cu-BP)

For isolation of Cu-binding peptides, aliquots of the soluble fraction from roots that had been exposed to the metal for 7 days were separated by gel filtration with Sephadex G-75 in 20 mM Tris-HCl (pH 8.0) containing 2 mM β-mercaptoethanol at a flow rate of 1.6 mL/min. Fractions (4 mL) were collected and their Cu concentration

and absorption at 254 and 280 nm were measured with an HGA 800 GF-Atomic Absorption Spectrometer (AAS; Perkin Elmer) and UV-VIS Spectrometer (Hewlett Packard), respectively were separated by gel filtration on Sephadex G-50, and 3 mL-fractions were collected. Elution was performed at a flow rate of 1.2mL/min. The column was calibrated with the following molecular weight standards: bovine serum albumin (66 kDa), rabbit metallothionein-1 (6.5 kDa), and Vitamin B_{12} (1.35 kDa). Eluted protein levels were monitored by absorption at 254 and 280 nm, and Cu concentrations were measured in the 3 mL fractions collected.

RESULTS AND DISCUSSION

Phytotoxicity Tests in soil

To examine the tolerance of herbaceous plants to Cu, the effects of various Cu concentrations on the rate of seed germination in soil was examined (Table 1).

The germination rates of *H. annuus*, *E. frumentacea*, and *C. communis* were not affected by the concentration of Cu in soil. In fact, the germination rate of *E. frumentacea* and *H. annuus* in Cu-contaminated soil (> 200 mg Cu/kg) was more than 90% of control. However, the germination rate of *M. sativa*, *A. aviennae*, and *Z. mays* was showed Cu dose-dependently reduction. The rate of germination of *M. savita* was significantly affected at concentrations above 50 mg Cu/kg.

Table 2 shows the effect of the Cu concentration on the root and shoot elongation of *C. communis*. The results show that the root and shoot elongation decreased as the Cu concentration increased. At 300 mg Cu/kg-soil, the root elongation of *C. communis* was 52% of control, although shoot growth was 96.2%. Thus, the roots of *C. communis* were more sensitive to Cu than the shoots.

We additionally found that there was a linear correlation of the fresh biomass and the Cu concentration in the soil (Table 2). The biomass was 60% of control at 300 mg Cu/kg-soil. High biomass is thought to result in high uptake and accumulation of heavy metals.

Table 2. Root length, shoot length and the biomass of *Commelina communis* grown in Cu-treatmented soil for 14 days. Units are mean±S.D

Concentration	0	50	100	200	300
	Cu (mg/L)				
Shoot (cm)	2.7±0.3	2.4 ±0.5	2.7 ±0.9	2.2 ±0.5	2.6 ±0.3
Root (cm)	2.5±0.5	3.0 ±1.5	3.2 ±0.7	1.8 ±0.4	1.3 ±0.4
Biomass (g)	0.8±0.01	0.07±0.02	0.08±0.02	0.07±0.02	0.07±0.02

Phytotoxicity Tests in water

To compare the tolerance of herbaceous plants to Cu, the effects of various Cu treatment on the rate of seed germination in water (Table 3).

At concentrations over 5 mg Cu/L-water, *A. avicennae* showed a similar germination rate as control plants. In addition, the germination rate of *C. communis* at 30 mg Cu/ L in hydroponic culture was 50% of control. Thus, *A. avicennae* and *C. communis* are not affected by the Cu concentration.

The results for the influence of Cu on root and shoot length, shown in Table 4, indicate that the length generally decreased with increasing Cu concentration. In addition, roots and shoots of *C. communis* eliminated over 52% and 80%, respectively, of the Cu from a solution of 5 mg $CuCl_2$/L. Furthermore, in the aquatic sys-

Table 3. Seed germination rate of plant species in Cu treatment water after 14 days

Concentration / Species	0	50	100	200	300
	Cu (mg/L)				
M. sativa	87.0± 3.0	80.0±10.0	90.0±10.0	50.0±20.0	47.0±13.0
A. avicennae	93.0± 7.0	90.0±10.0	80.0±10.0	90.0±10.0	80.0±20.0
C. communis	87.0± 3.0	70.0±20.0	63.0± 7.0	65.0±15.0	67.0±13.0
E. frumentacea	80.0±10.0	70.0±10.0	50.0±10.0	50.0±20.0	30.0±10.0
Z. mays	87.0±13.0	53.3± 5.8	47.0±13.0	40.0±10.5	13.5± 6.5
H. annuus	70.0±20.0	80.0±10.0	70.0±20.0	70.0±20.0	60.0±10.0

Table 1. Seed germination rate of plant species in Cu treatment soil after 14 days

Concentration / Species	0	50	100	200	300
	Cu (mg/kg)				
Medicago sativa	100.0±10.0	60.0±10.0	60.0±10.0	67.0±13.0	64.0± 6.0
Abutilon avicennae	80.0±10.0	98.0± 0.0	93.3± 5.8	91.0±11.0	30.0±10.0
Commelina communis	85.0± 7.2	90.0±10.0	93.3± 5.8	83.7± 5.5	70.0±20.0
Echinochloa frumentacea	80.0± 0.0	93.3± 0.0	95.5± 4.5	93.3± 5.8	100.0±20.0
Zea mays	79.0± 0.0	85.0±10.0	83.3±11.5	75.3± 9.5	66.6±11.5
Helianthus annuus	100.0± 0.0	93.3± 0.0	100.0±10.0	93.3± 5.8	90.0±20.0

Table 4. Root length, shoot length and the biomass of *Commelina communis* grown in Cu-treatmented water for 14 days. Units are mean ± S.D

Concentration	0	5	10	20	30
	Cu (mg/L)				
Shoot (cm)	13.6 ±0.3	2.5 ±0.2	2.2 ±0.7	2.0 ±0.3	1.7 ±0.1
Root (cm)	5.47±0.2	3.03±0.3	2.7 ±0.2	2.2 ±0.4	1.8 ±0.1
Biomass (g)	0.25±0.01	0.09±0.01	0.07±0.01	0.02±0.001	0.01±0.005

tem, Cu hampered the development of shoots more than roots.

We also found that the biomass of *C. communis* decreased almost linearly over a 14-day period when grown in a Cu containing solution (Table 4). In particular, there was a 50% reduction of biomass in *C. communis* when grown in a solution containing 10 mg Cu/L. This also caused a 73% reduction in elongation. Collectively, these studies in the aquatic system show that Cu hampers shoot development more than root development. Furthermore, the roots, shoots, and biomass are more sensitive to Cu-contaminated soil than Cu-contaminated water.

Absorption and uptake of Cu in soil by *C. communis*

To test whether *C. communis* removes Cu from the soil, we measured the soil Cu content after 30 days of growth in Cu-contaminated soil. The change in Cu levels in Cu-contaminated soil over a 30-day period of plant growth is shown in Fig. 1. Plant growth was reduced with Cu content. In addition, compared to soil with no plants, soil containing *C. communis* plants had a reduction in exchangeable Cu. Specifically, in the presence of the plants, there was a 41% reduction in Cu in soil containing 50 mg Cu/kg and a 53% reduction in soil containing 100 mg Cu/kg.

In soil containing 200 mg Cu/kg, *C. communis* reduced the Cu content 35%, but even without plants, there were reductions of 37%, 37%, and 20% from soil containing 50, 100, and 200 mg Cu/kg soil, respectively. In 300 mg Cu/kg soil, the removal rate with plants was 9% and, without plants, 3%. Furthermore, exchangeable and soluble Cu were decreased compared to the initial soil, but there was not a significant difference between soil with and without plants. Cu is transported in soil more easily if it is chelated and is more easily absorbed from soil if it is chelated (White *et al*. 1991).

The reduction of Cu in the soil by *C. communis* was most significant in soil containing 50 or 100 mg Cu/kg. We therefore measured the accumulation of Cu by *C. communis* (Fig. 2). Accumulation of Cu in the roots and shoots increased as the level of Cu contamination was raised. In addition, the accumulation of Cu in the roots was three- to four-fold higher than in the shoots. This higher accumulation in root compared to shoot is consistent with previous reports (Baker 1981).

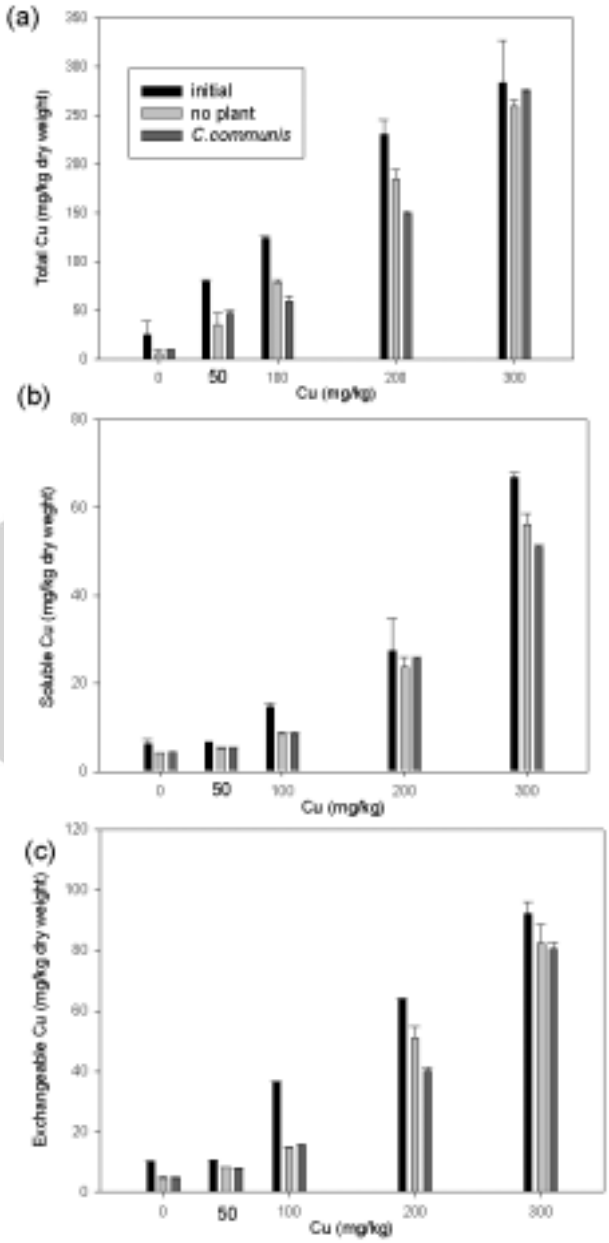

Fig. 1. The changes Cu amount of Total-(a) Soluble-(b), Exchangeable -(c) Cu amount (mg/ kg dry matter) in each soil.

Cu uptake by *C. communis* in a hydroponic system

To examine the uptake of Cu from water, we cultured *C. com-*

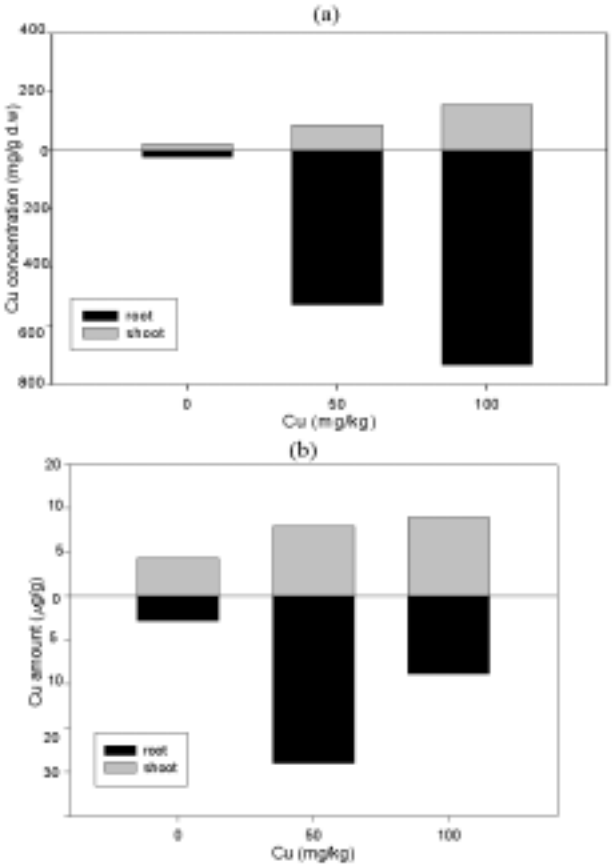

Fig. 2. Cu concentration (a) and amounts (b) in *Commelina communis*.

munis for 5 weeks in water contaminated with Cu (Fig. 3). We found that the uptake of Cu by *C. communis* increased with increasing concentration of Cu in the water. The maximal uptake of Cu was from water containing 20 mg Cu/L.

To confirm the correlation between accumulated metal and metal

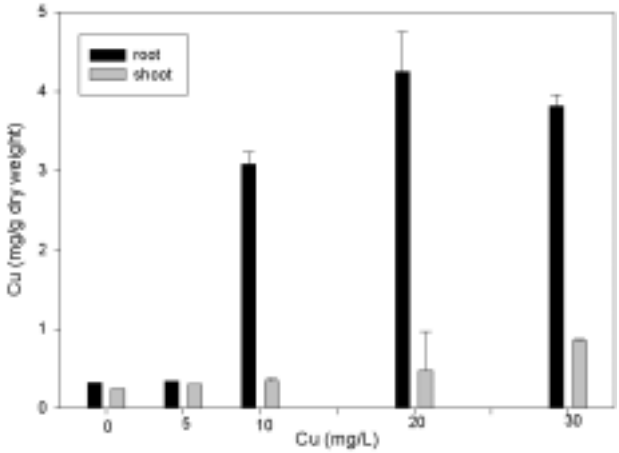

Fig. 3. Uptake of Cu by *Commelina communis* in different treatmented solution 0, 5, 10, 20, 30 mg Cu/L.

available in the Hoagland solution, *C. communis* specimens were exposed to 10 mg Cu/L and 20 mg Cu/L for 0, 3, 5, or 7 days. The maximal rate of uptake by *C. communis* was examined in plants grown in water containing 10 or 20 mg Cu/L (Fig. 4).

The amount of Cu taken up by the roots from water containing 20 mg Cu/L was twice as high as from water containing 10 mg Cu/L. After 7 days, the amount of Cu taken up by the roots and shoots did not increase. Approximately 2.8 and 1.5 mg Cu/g was accumulated in the roots and shoots, respectively, when *C. communis* exposed to water containing 10 mg Cu/L. Exposure to 20 mg Cu/L resulted in accumulation of 4.9 and 1.2 mg/g in the roots and shoots, respectively. Therefore, we expect that 1 ton of *C. communis* would be able to remove 20.7 kg Cu. These results suggest that hydrophytes can remove Cu from soil or water.

Isolation of a Cu-Binding peptide

To isolate the Cu-binding ligand, we performed gel filtration

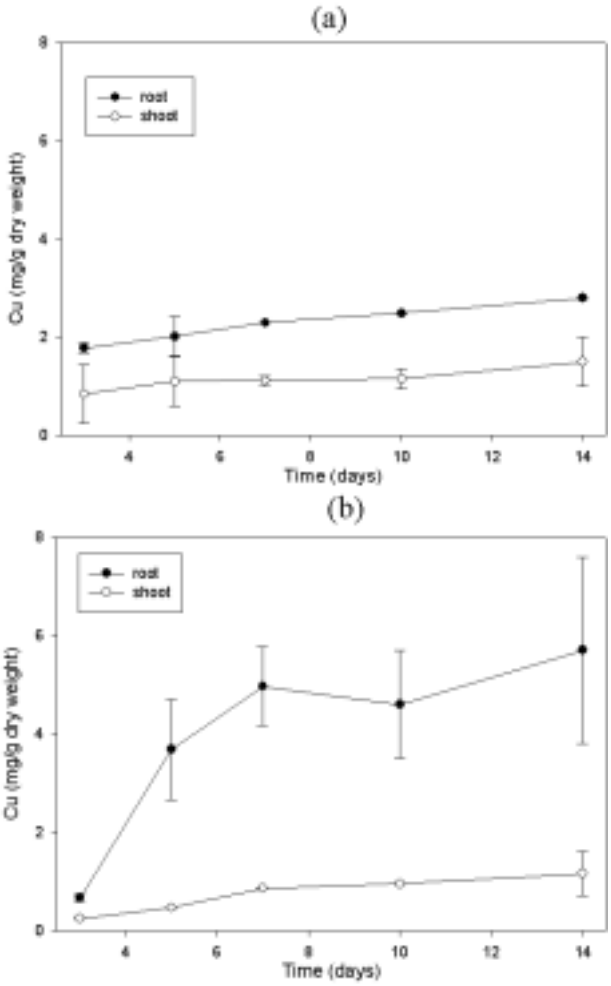

Fig. 4. Uptake of Cu by *Commelina communis* in 10mg/L Cu solution (a), 20mg/L Cu solution (b) for 14 days.

chromatography (Sephadex G-75) on the cytosolic (soluble) fraction from plants that had been exposed Cu for 7 days (Fig. 5). In the control group, we did not observe a Cu-binding fraction. In plants exposed to 10 mg Cu/L, we observed two Cu peaks in the chromatogram. The small peak (fractions 17 to 25) was observed at ~60 kDa, and was referred to as the high molecular weight ligand (HMWL), and the large peak (fractions 51 to 67) was observed at ~1 kDa and was referred to as the low molecular weight ligand (LMWL). The HMWL complex has not been a common feature in the extracts of plant cells exposed to metals. However, Eanetta and Steffens (1989) isolated sulfite in phytochelation(PC)-metal complexes in tomato cells. Binding of Cu and Cd to a HMWL of approximately 13 kDa isolated from *Silene vulgaris* and tomato cells has been reported by Leopold *et al*. (1999). Complexes of Cd and HMWLs from *Silene vulgaris* (Verkleij *et al*. 1990) and Indian mustard (*Brassica juncea*) (Speiser *et al*. 1992) have also been reported. The <1 kDa peptide, the ligand containing most Cu, is estimated as phytochelation used to isolate at different higher plants. Grill *et al*. (1987) also isolated a low molecular weight ligand (2.2 kDa) that binds to Cd in the leaves of *Brassica oleracea* after Cd exposure. In *Datura innoxia* (Robinson *et al*. 1987) a large percentage of Cd (>80%) is bound to low molecular weight peptides, and in maize (Leblova and Stiborova 1986) about 72% of the Cd is bound to such peptides (possibly (*r*-EC)nG). In the present work, when the soluble fraction from soybeans exposed to Cd was separated by gel filtration on Sephadex G-75, a low molecular weight ligand was identified at 2.2 kDa, indicating the presence a small metal-binding peptide similar to that found in *C. communis*.

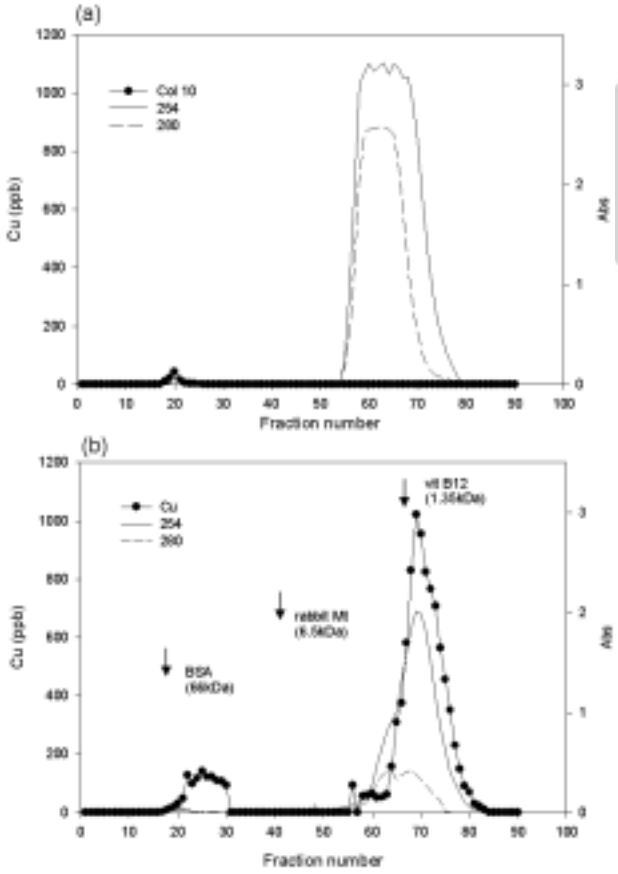

Fig. 5. Elution profile of gel-chromatography on Sephadex G-50 of untreated (a), copper treated (b) from *Commelina communis* roots.

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(*Received January 17, 2005; Accepted February 17, 2005*)