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ORIGINAL RESEARCH

Characterization of Cadmium-Binding Ligands from Roots of *Echinochloa crusgalli* var. *frumentacea*

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Abstract Herbaceous *Echinochloa crusgalli* var. *frumentacea* is highly resistant to a wide range of heavy metal concentrations. However, its detoxification mechanism has not been reported yet. We exposed plants to 100 μM $CdCl_2$ for 7 days then examined cadmium accumulation and its subcellular distribution in binding to ligands. Cd concentration increased over the exposure period to a saturation point at day 5 when its level reached 1,732.41 μg g^{-1} dry weight, an amount about 820 times greater than that found in the control. In the roots, most Cd was distributed in the insoluble fraction, perhaps because of an absorption mechanism at the root surface. Our profile for distribution revealed two Cd-binding ligand peaks: a high molecular weight of 60 kDa and a 2.5-kDa Cd-binding ligand. The latter increased with time and had a typical phytochelatin (PC) amino acid composition of predominantly cysteine, glutamate, and glycine (16.5%, 16.6%, and 11.9%, respectively). The ratio of glutamate/cysteine/glycine was 1.4:1.4:1.0, which is similar to that for other typical PCs.

Keywords Amino acid · Cadmium · Detoxification · *Echinochloa crusgalli* var. *frumentacea* · Heavy metal · Phytochelatin

Plants growing near industrial, shooting, or waste disposal sites and on some natural environments take up lethal levels of toxic metals such as Cd, Ni, Pb, and Hg. While the growth and metabolism of most species are severely restricted under such conditions, some are tolerant to toxic levels (Steffens 1990). These plants have mechanisms that sequester metals and, thereby, achieve cellular metal homeostasis and detoxification (Rauser 1995). Several reports have described the development of metal-binding ligands, including metallothionein (MT; Leblova et al. 1986; Robinson et al. 1993), and metal-binding polypeptides known as phytochelatins (PCs; Grill et al. 1987).

Such PCs as cadystins, Cd peptides, or γ-glutamyl peptides are nonprotein metal-binding polypeptides that possess the unusual structure of $(\gamma Glu\text{-}Cys)_n Gly$, where $n = 2$ to 11. Although the heavy metals Cd, Pb, Zn, Ag, Ni, Hg, Cu, and Sn are primary inducers, the strongest is cadmium. Current research has focused on plants that induce PCs for phytoremediation of soils contaminated with Cd (Brown et al. 1995; Salt et al. 1995).

Echinochloa crusgalli var. *frumentacea* is an indigenous Korean herb that is highly resistant to a wide range of heavy metal concentrations (Chang et al. 2001). However, its cellular detoxification mechanism has not yet been reported. Here, we purified a Cd-binding ligand (Cd-BL) from the roots of *E. crusgalli* and characterized its amino acid composition. Our objective was to determine its role in plant tolerance to Cd in toxic environments.

Materials and Methods

Plant Materials and Cadmium Treatment

Plants of *E. crusgalli* were grown for 4 weeks in clean soil in a greenhouse maintained at 25°C. They were then transferred from their pots into Hoagland solution and

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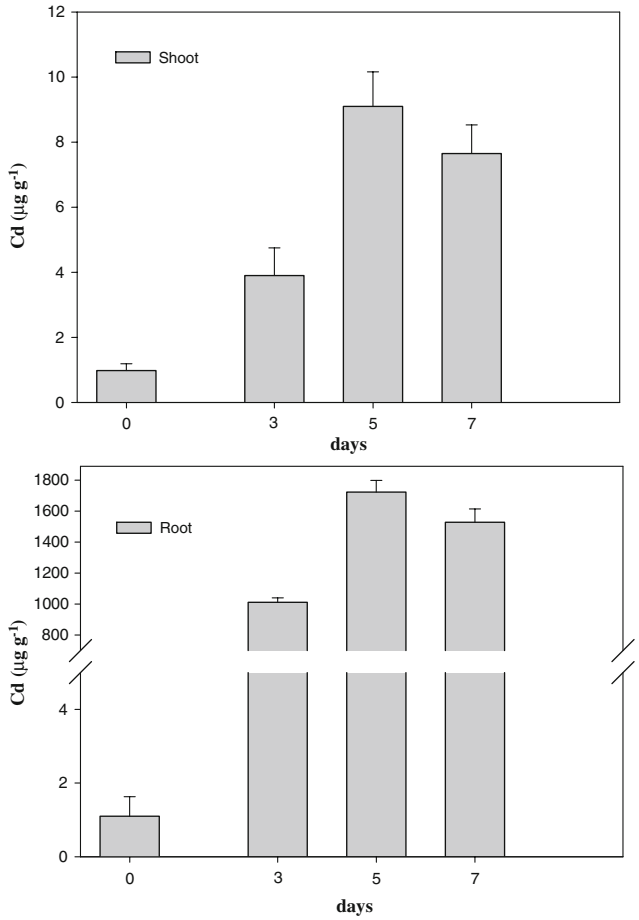

Fig. 1 Distribution of Cd (μg g^{-1} dry weight) between **a** shoot and **b** root fractions of *E. crusgalli* exposed to 100 μM $CdCl_2$. Values are means of three replicates (n = 9)

grown hydroponically at 25°C with aeration. After 7 days of adaptation, they were added 100 μM $CdCl_2$ to the Hoaglands for a further 7 days.

Metal Analysis

Control and cadmium-treated plants were analyzed for 3, 5, and 7 days. At those intervals, they were washed with distilled water for 2 min and oven-dried at 70°C before being divided into their shoot and root portions and weighed. To determine the Cd concentration in each subcellular fraction, roots were rinsed in cold distilled water for 2 min, blotted, and frozen at –80°C. The masses were pulverized in liquid N_2 with a pestle before 10-g samples were homogenized in Buffer A (20 mM Tris–HCl buffer (pH8.0) containing 2 mM β-mercaptoethanol) and digested in 65% HNO_3.

Aliquots of the homogenate were centrifuged at 20,000×g for 30 min at 4°C. The supernatant was denoted "soluble fraction", while the pellet was designated "insoluble fraction". Cadmium levels in both soluble and insoluble fractions were measured by acid digestion as described above. An aliquot of the soluble fraction was applied to a gel-filtration column. Cadmium concentrations in the eluate derived from gel-filtration chromatography were measured directly by graphite AAS.

Purification of Cadmium-Binding Ligands

Aliquots of the soluble fractions were applied to a Sephadex G-50 column equilibrated with Buffer A, and 3-ml fractions were collected. Elution was performed at a flow rate of 0.8 ml min^{-1}, and Cd concentrations were measured in all fractions.

The column was calibrated with the following molecular weight standards: bovine serum albumin (66 kDa), rabbit metallothionein (MT)-1 (6.5 kDa), and vitamin B_{12} (1.35 kDa). Eluant protein levels were monitored at A_{254} and A_{280}. All cadmium-binding peptide fractions were pooled, applied to a DEAE-Sepharose column (1.5 × 3 cm) equilibrated with Buffer A, and washed with the same buffer. Bound proteins or peptides were eluted at pH8.0 over a linear gradient (20 to 200 mM Tris–HCl) in a buffer containing 2 mM β-mercaptoethanol.

Amino Acid Analysis

Aliquots of purified peptides were de-salted on a dialysis membrane (Spectra/Por® CE, MWCO 500; Spectrum) with several buffer changes of distilled water. To detect their phenylisothiocyanatyl derivatives, peroxidized samples were hydrolyzed in HCl and analyzed by reverse-phase

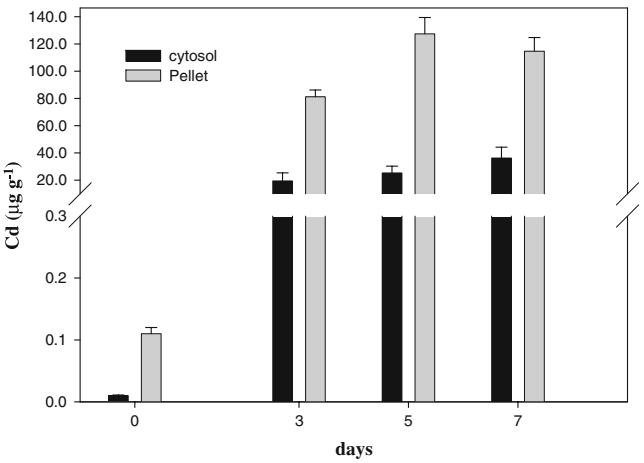

Fig. 2 Partitioning of Cd (μg g^{-1} wet weight) between pellet and cytosol fractions from *E. crusgalli* roots. Values are means of three replicates (n = 9)

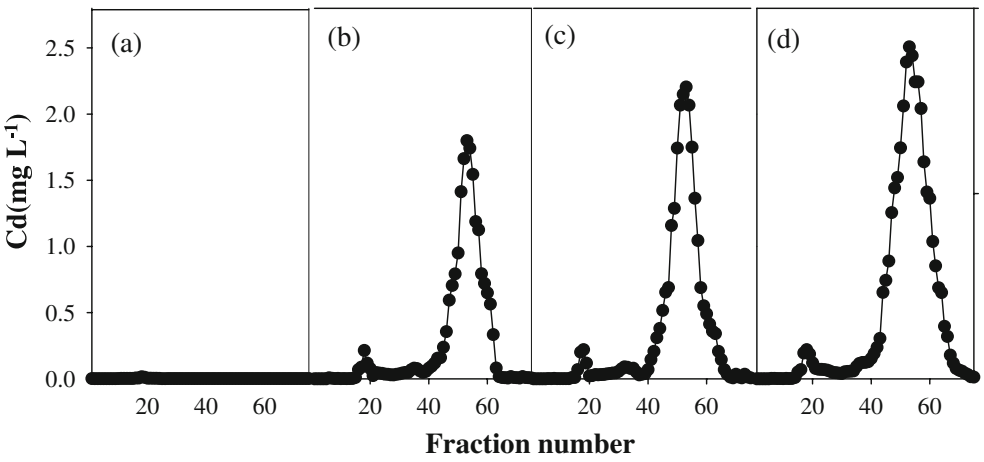

Fig. 3 Size-exclusion chromatography (Sephadex G-50) for soluble fraction of *E. crusgalli* roots from control (**a**) or after exposure to Cd for 3 days (**b**), 5 days (**c**), or 7 days (**d**)

HPLC (Hewlett-Packard 1100) on an Eclipse WDB C18 column (Hewlett-Packard).

Results and Discussion

To examine the correlation between toxin accumulation and available metals in Hoagland solution, plants of *E. crusgalli* were exposed to 100 μM $CdCl_2$ for 7 days. Cadmium concentrations increased gradually in the first 5 days when they reached a saturation point (Fig. 1), with values of 1,540 times higher in the roots and about nine times higher in the shoots compared with the untreated control. Therefore, the rare phenomenon of Cd hyperaccumulation in this species was confirmed by our hydroponic experiments (Fig. 1).

To investigate the distribution of cadmium within the plant, we analyzed shoot and root portions separately (Fig. 1). In the control, Cd concentrations were nearly the same in both tissue types, whereas in the treatment samples, the roots bound up to 99% of that metal. Jarvis et al. (1976) have reported that the order of relative Cd accumulation is root → stem → leaf. Namely, nutrients in the soil are initially absorbed through the root cell walls. We also determined the subcellular partitioning of cadmium between soluble and insoluble fractions in the roots (Fig. 2). In the control, more than 91% of the metal was detected in the soluble fraction, whereas in the treated plants, about 80% was distributed in the insoluble fraction. This activity likely would be an important component within a phytoremediation process, influencing the absorption of toxins at the root surface (Salt et al. 1995).

To identify the ligands involved in binding and sequestering of cadmium in the cytosol, we performed gel-filtration chromatography with the soluble fraction from plants that had been exposed to Cd for 0, 3, 5, or 7 days (Fig. 3). Two peaks were revealed: a high-molecular-weight ligand (HMW; 60 kDa) and a Cd-binding ligand (Cd-BL; 2.5 kDa). Most of the Cd (about 97%) was bound to the Cd-BL (Table 1). Although the height of that peak did not change over time, the level of Cd-BL did, possibly because the ligand maintained a constant Cd influx while the metal was accumulating in

Table 1 Concentration of Cd in HMW and Cd-BL fractions obtained from gel-filtration chromatography

Exposure time (days)	HMW		Cd-BL	
	μM	%	μM	%
0	<0.005	–	–	–
3	0.0467	3.12	1.45	96.88
5	0.0519	2.70	1.87	97.30
7	0.0805	2.84	2.75	97.16

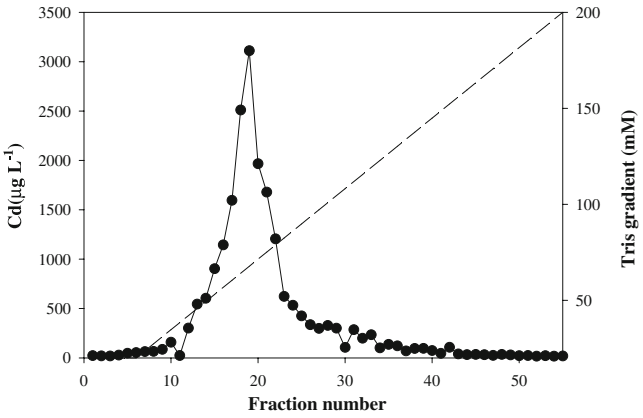

Fig. 4 Anion-exchange chromatography (DEAE-Sepharose) of Cd-binding ligand derived from fractions of size-exclusion chromatography (see Fig. 1) showing Cd concentration in gradient of solvent

Table 2 Amino acid composition of Cd-BL derived from roots of *Echinochloa crusgalli*

Amino acid	% of total residues
Ala	7.9
Arg	2.9
Asp/Asn	3.1
Cys	16.5
Glu/Gln	16.6
Gly	11.9
His	1.1
Ile	9.3
Leu	4.6
Lys	5.6
Met	1.1
Phe	1.4
Pro	3.0
Ser	3.7
Thr	2.1
Tyr	4.2
Trp	0.0
Val	5.0
Total	100

the cytosol. HMW Cd complexes have also been reported from *Silene vulgaris* (Verkleij et al. 1990) and *Brassica juncea* (Speiser et al. 1992), but are not a common feature in the extracts of plant cells exposed to metals (Eanetta and Steffens 1989). The 2.5-kDa peptide, the ligand containing the most Cd, is proposed to be the phytochelatin found in various higher plants. Using a Sephadex G-50 column, Grill et al. (1987) also have isolated a 2.2-kDa low-molecular-weight (LMW) ligand that binds cadmium in the treated leaves of *Brassica oleracea*. Likewise, in *Datura innoxia* and in maize, >80% and about 72%, respectively, of the Cd is bound to LMW peptides (possibly $(\gamma\text{-EC})_n$G; Robinson et al. 1987).

To isolate and identify this Cd-BL, Cd-containing fractions (numbers 47 to 61; Fig. 3d) were pooled and purified by ion exchange chromatography (Fig. 4). One peak was eluted at a 68 mM Tris concentration. The amino acid composition of Cd-BL (Table 2) was dominated by cysteine (16.5%), glutamate (16.6%), and glycine (11.9%). Its ratio of 1.4:1.4:1.0 for glutamate, cysteine, and glycine is similar to the range of 2:2:1 to 11:11:1 reported for typical PCs (Grill and Zenk 1985). Taken together, these results suggest that our Cd-BL is a phytochelatin, a ligand that is involved in detoxifying heavy metals (Jackson et al. 1987). We also conclude that *E. crusgalli* induces PCs as a cellular defensive mechanism, acting against the heavy burden of cadmium in that species. Because the Cd-BL was gradually induced over the exposure period and sequestered most of the Cd in the cytosol, it might serve as a biochemical tool for assessing bioavailable metal concentrations at industrial and shooting sites contaminated with cadmium. Our study suggests that sorption is the most important mechanism toward increasing PC resistance and invites further research that compares this trait with that in other Cd-hyperaccumulating plants.

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