J. Plant Biol. 40 (4) : 275~278 (1997)

Effect of Spermine on the Phytochelatin Concentration and Composition in Cadmium-treated Roots of *Canavalia lineata* Seedlings

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Effect of spermine on the phytochelatins (PCs) in cadmium-treated roots of *Canavalia lineata* seedlings was studied. With the treatment of spermine, total nonprotein thiol (SH) contents decreased by 55% in roots of Cd-treated plants. Glutathione (GSH) synthetase activity was inhibited by 36.8% in roots and cysteine synthase was also inhibited by 9.5% while γ-GluCys synthetase activity was not affected. From the PC-Cd complex analyses by gel column chromatography, it was found that Cd+spermine-treated roots contain an additional PC that has low affinity for Cd, in addition to Cd-induced PC whose SH:Cd ratio is 1:1. Spermine affected the PC concentration and composition in the Cd-treated roots of *C. lineata* seedlings.

Keywords: Cadmium, *Canavalia lineata*, phytochelatins, spermine

Phytochelatins (PCs) that have the structure (γ-Glu-Cys)nGly, where n=2~11, are synthesized in plants with evolved tolerances to heavy metals (Rauser, 1990; Steffens, 1990). The structure of PCs indicates that these peptides are nonprotein thiol (SH) compounds synthesized enzymically and are not primary translation products of mRNAs (Hell and Bergmann, 1990; Ruegsegger and Brunold, 1992). γ-GluCys synthetase and glutathione (GSH) synthetase are involved in the synthesis of these heavy metal binding peptides (Hayashi *et al.*, 1991; Ruegsegger and Brunold, 1992; Ruegsegger *et al.*, 1990). PCs have been considered to play a role in metal tolerance as a naturally or artificially selected heritable increase in the capacity to tolerate higher concentrations of metal exposure. PCs may be good quantitative indicators of heavy metal impacts in environments. Increased tolerance has been suggested to result from overproduction of PCs (Bennetzen and Adams, 1984; Steffens *et al.*, 1986). PCs are also involved in the cellular homeostasis of biologically essential metals (Grill *et al.*, 1987; Kneer and Zenk, 1992). The role for PCs in metal detoxification is substantiated by the fact that inhibition of PC synthesis, either through sulfur starvation or by treatment with buthionine sulfoximine, increases the sensitivity of plants and cell suspensions to heavy metals, regardless of whether biologically essential or nonessential metals are concerned (Choi *et al.*, 1996; De Vos *et al.*, 1992; Howden and Cobbett, 1992). PCs form complexes with the toxic heavy metals which are sequestered in the vacuole (Salt and Rauser, 1995; Vogeli-Lange and Wagner, 1990).

Cadmium (Cd) is the strongest inducer in PC synthesis among the common metals. The molecular weights and SH:Cd ratios of PC-Cd complexes varied 1.7 to 13.8 kDa and 1:1 to 3:1, respectively, according to plant species, plant tissues, culture conditions, Cd concentrations, treatment time, extract conditions, and chromatography conditions (Grill *et al.*, 1986; Inoue *et al.*, 1994; Rauser and Meuwly, 1995; Reese *et al.*, 1988). In *Canavalia lineata* seedlings, PCs were synthesized by Cd, and the molecular weights and SH:Cd ratios of PC-Cd complexes are different between leaves and roots (Choi *et al.*, 1996). Although PCs may be involved in many aspects of plants, there

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are many challenges to be met in reaching a full understanding of the ways PCs control the metal impacts. Polyamines are found in all organisms, in a wide variety of capacities. In plants they are implicated in the regulation of developmental processes such as embryogenesis, senescence, and flowering (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1995). It was reported that polyamines affect the enzyme activities in plants (Altman, 1982; Kaur-Sawhney and Galston, 1979). Therefore, we have examined the effects of polyamine on the biosynthetic enzyme activities, concentrations, and SH:Cd ratio of PC in the seedlings of *C. lineata* during the PC induction by Cd.

MATERIALS AND METHODS

The germination and culture conditions of *Canavalia lineata* seedlings have been described previously (Choi *et al.*, 1996). However, in this experiment, seedlings were treated with Cd for 4 days. On the contrary, Choi *et al.* (1996) treated for 5 days. The total nonprotein thiols were measured by the method of De Vos *et al.* (1992). The Cd content in the extracts was measured by an atomic absorption spectrophotometer, GBC model 904. Activities of γ-GluCys synthetase and glutathione (GSH) synthetase in cell-free extracts were assayed by measuring the formation of γ-GluCys or GSH by HPLC as mentioned in Choi *et al.* (1996) and cysteine synthase activity was determined by the method of Nakamura *et al.* (1987). PC-Cd complexes were analysed as described in Choi *et al.* (1996). Spermine was assayed by HPLC after derivatization with benzoyl chloride (Flores and Galston, 1982).

RESULTS AND DISCUSSION

The effect of spermine on the phytochelatins (PCs) was examined in the *Canavalia lineata* seedlings treated with Cd (Table 1). After spermine addition for 4 days, the thiol (SH) content was lower than control by 55% in roots. The inhibition was increased with increase of the incubation time. It is known that polyamines have been implicated in an overwhelming array of plant growth and developmental processes (Evans and Malmberg, 1989). Other polyamines, putrescine and spermidine, also inhibited the accumulation of phytochelatins (data not shown). However the inhibition effects of putrescine and spermidine were relatively low as compared with that of spermine, and thus spermine was used in this experiment.

The effect of spermine on the activities of cysteine synthase, γ-GluCys synthetase, and glutathione (GSH) synthetase was examined. The γ-GluCys synthetase activity was not affected (data not shown). But GSH synthetase activity was inhibited by 36.8% in roots, and cysteine synthase that synthesizes cysteine, a precursor of γ-GluCys, was also inhibited by 9.5% (Table 2). From these results, it is assumed that the

Table 1. Effect of spermine (Spm) on the phytochelatins in roots of Cd-treated *C. lineata* seedlings

Treatment	Phytochelatins (thiol, μmol/g f.w.) Incubation time, days				
	0	1	2	3	4
Cd	0.440±0.10	0.869±0.07	1.260±0.27	1.430±0.47	1.950±0.07
Cd+Spm	-	0.670±0.09	0.680±0.23	0.840±0.21	0.870±0.41

Eleven day-old seedlings were transferred to the Cd (50 μM) and Cd+Spm (5 μM) treated media. Data are the mean±SD of three independent experiments.

Table 2. Effect of spermine (Spm) on the activities of GSH synthetase and cysteine synthase in roots of Cd-treated *C. lineata* seedlings

Treatment	GSH synthetase, GSH nmol/min/mg protein		Cysteine synthase, Cys mol/min/mg protein	
	Days		Days	
	0	2	0	2
Cd	2.20	1.90	0.524	0.420
Cd+Spm	-	1.20 (36.8)	-	0.380 (9.50)

13-day-old plants exposed to 50 μM Cd for the last 2 days were transferred to the Cd (50 μM) and Cd+Spm (5 μM) treated media. Data represent the mean of two separate determinations. Percent inhibition compared to controls without spermine is given parentheses.

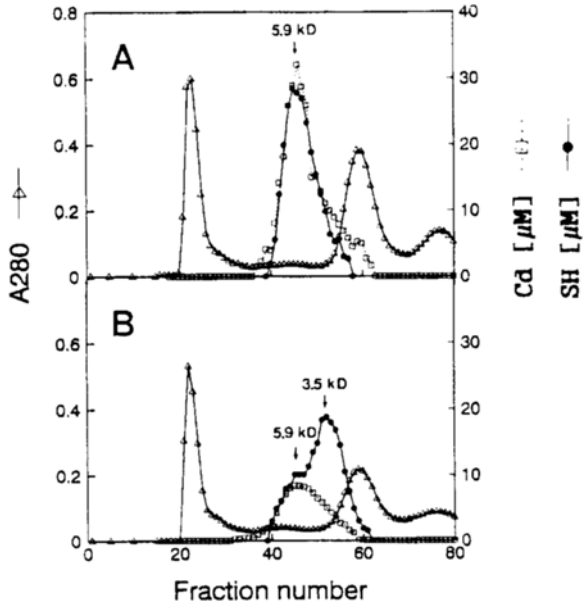

Fig. 1. Sephadex G-50 gel column chromatography profiles of crude extracts from roots of 15-day-old plants exposed to 50 μM Cd for the last 4 days (A) and 50 μM Cd for the last 4 days and 5 μM spermine for the last 2 days (B). The Cd ratios to SH in fractions 46-55 : 1.15, 1.07, 1.11, 0.80, 0.98, 1.03, 1.13, 1.55, 1.69, 1.41 (A); 1.16, 0.91, 0.75, 0.60, 0.44, 0.26, 0.22, 0.16, 0.15, 0.13 (B).

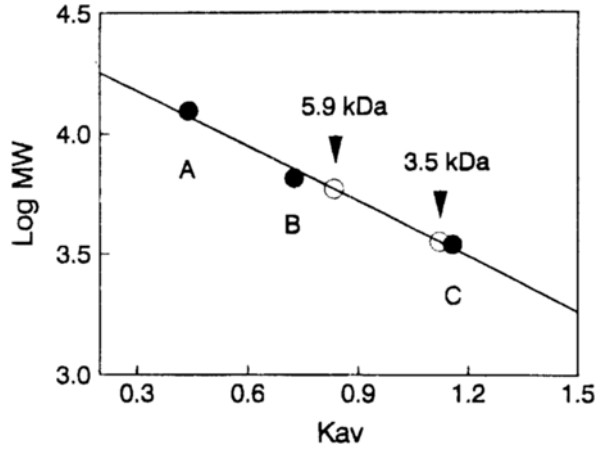

Fig. 2. Molecular weight determinations of PC-Cd complexes in roots by Sephadex G-50 gel column chromatography. Calibration standards: A, cytochrome C (12.4 kDa); B, aprotinin (6.5 kDa); C, insulin chain B (3.45 kDa). This result was obtained with the thiol fractions of Fig. 1. Cd-treated roots (Fig. 1A) contained 5.9 kDa complex, and Cd+ spermine-treated roots (Fig. 1B) contained 5.9 and 3.5 kDa complexes.

thiol content decrease in the roots of Cd-treated *C. lineata* by spermine is mainly due to the reduction of PC synthesis by the inhibition of GSH synthetase activity. However, to our knowledge, this interesting aspect that PC synthesis can be reduced due to the inhibition of enzyme activity by spermine has not been reported.

In order to examine whether spermine induces the alterations of PC-Cd complexes in the roots, the complexes were extracted from the roots, separated by Sephadex G-50 gel column chromatography and characterized (Fig. 1 and 2). The molecular weight of PC-Cd complex was 5.9 kDa (Fig. 2) and the SH:Cd ratio was 1:1 in the spermine-nontreated roots (Fig. 1A). In the elution profile, Cd was eluted in the PC peak. This result was different from a previous study concerning the molecular weight, but was identical in the SH:Cd ratio (Choi *et al.*, 1996). It was reported that the complexes are variable in size (1.7-13.8 kDa) according to the plant species and experimental conditions (Grill *et al.*, 1986; Rauser and Meuwly, 1995; Reese *et al.*, 1988). In this experiment, Cd treatment time was 4 days, in contrast with 5 days from the previous work (Choi *et al.*, 1996). Other experimental conditions were identical with Choi *et al.*, (1996). The reasons of PC-Cd complex alterations in accordance with Cd treatment time were not understood. On the other hand, the two peaks of PC complexes were detected in the roots treated with spermine (Fig. 1B), but the two peaks were not isolated completely by chrpmatography. The molecular weights of PC-Cd complexes from the spermine treated roots were 5.9 kDa and 3.5 kDa (Fig. 2). In the spermine treated roots, an additional PC of 3.5 kDa, which was not detected in the control, was recognized (Fig. 1). As shown in Fig. 1, B, the Cd peak overlapped with the peak of 5.9 kDa complex, whereas no Cd was detected in the 3.5 kDa complex. It seems that the new 3.5 kDa complex either has a much higher ratio of SH:Cd or does not bind Cd. It was reported that PCs possess variable affinities for Cd (Hayashi and Nakagawa, 1988).

Because of the protonated amino and imino groups at physiological pH, polyamines can bind to a variety of anionic cell constituents such as nucleic acids, phospholipids, negatively charged protein residues of membranes, and pectic substances of cell walls (Evans and Malmberg, 1989; Galston and Kaur-Sawhney, 1995). Thus, in order to examine whether the new PC can form a complex with spermine or not, spermine was analysed with the fractions of gel column chromatography by HPLC (data not shown). As spermine was not detected in the fractions, it is assumed that PCs do not form complex with spermine.

In conclusion, spermine affected the Cd-treated roots of *C. lineata* seedlings in PC concentration and composition. The reasons are not understood; however, it is suggested that polyamines may adversely affect the plants that are situated at the heavy metals. Polyamine increase may cause plants not to be adapt to the heavy metal impacts. Therefore, the interactions between polyamines and PC synthesis should be studied more carefully.

ACKNOWLEDGMENTS

This research was supported by the Basic Science Research Institute Program, Ministry of Education, 96-project No. BSRI-96-4408 and in part by the Korea Science and Engineering Foundation (KOSEF) through the Research Center for Cell Differentiation at Seoul National University (96-5-1).

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Received September 9, 1997
Accepted October 31, 1997