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THE FORMATION OF Cd–PHYTOCHELATIN COMPLEXES IN PLANT CELL CULTURES

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Key Word Index—*Rauvolfia serpentina*; *Silene cucubalus*; Cd–phytochelatin complexes; sulphide; formation and processing of Cd–phytochelatin complexes.

Abstract—Three distinct Cd–phytochelatin complexes were purified to apparent homogeneity by gel filtration on Sephadex G50 of crude Cd–phytochelatin preparations isolated from Cd-treated suspension cultures of *Rauvolfia serpentina*. These purified complexes were analysed and found to be significantly different in PC chainlength, S:Cd ratio, and sulphide content. Kinetic measurements of PC_n and sulphide content in Cd-treated suspension cultures of *R. serpentina* and *Silene cucubalus* showed that there are two possible ways of decreasing the amount of sulphur (and nitrogen) needed for Cd^{2+} sequestration: either by increasing the PC chainlength or by incorporating sulphide into the complexes. The latter process appears to be the more effective. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Plants detoxify intracellular heavy metal ions by complexation with phytochelatins, which are small peptides that are synthesized by a heavy metal activated γ-glutamylcysteine dipeptidyl transpeptidase (PC synthase) from glutathione [1]. Very few structural details are known about these Cd–PC complexes, mainly due to the fact that all crystallization efforts have failed to date [2]. In contrast, metallothioneins (MT), the heavy metal detoxifying proteins of animals and some fungi, are well characterized down to X-ray structure [3]. The most elaborate suggestion of the complex structure of a Cd–PC complex is derived from extended X-ray absorption fine structure (EXAFS) spectroscopy [4]. These authors examined a crude Cd–PC preparation from suspension cultures of *Rauvolfia serpentina*. They found a sole Cd–thiolate coordination with a Cd–S bond length of 2.52±0.02 Å. All carboxylate groups face the outside of the complex, explaining the polyanionic, extremely hydrophilic character of Cd–PC isolates. Previously, Grill *et al.* [5] found two different complexes with apparent MWs of 2.5 and 3.6 kDa in Cd-treated suspension cultures of *R. serpentina*. Subsequently, the occurrence of two different Cd–PC complexes was reported from *Schizosaccharomyces pombe* [6], *Euglena gracilis* [7] and *Candida glabrata* [8]. Kondo *et al.* [6] analysed both complexes from *S. pombe* and found PC_2 mainly in one complex, whereas the other consisted mainly of PC_3. Acid-labile sulphur (S^{2-}) was found as ligand in Cd–PC complexes from *S. pombe* [9, 10], *E. gracilis* [7], *C. glabrata* [8], *Lycopersicon esculentum* [11], *Silene cucubalus* [12] and *Brassica juncea* [13], but sulphide was found to a significant extent only in the higher MW complex [9, 13]. While metallothioneins generally show a 3:1 ratio of S:Cd, varying values, ranging from 2:1 [14] to 3:8:1 [4], were found for the phytochelatins. In Cd–PC from *C. glabrata* [8] or tomato cells [15] Cd–sulphide semicrystallites were found. Therefore, sulphide appears to play a significant role in Cd-detoxification of many organisms. Recently, three different Cd–PC complexes in *R. serpentina* [16] and *S. pombe* [17] could be demonstrated upon Cd^{2+}-treatment. In this paper, we report on the isolation and characterization of these complexes from *R. serpentina* suspension cultures as well as a high-MW (HMW) complex from *S. cucubalus* cultures.

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RESULTS

Cd–PC from *R. serpentina* separated under strict conditions yielded three distinct peaks upon Sephadex G50 chromatography [16]. For further characterization of these Cd–PC complexes, the separation procedure had to be modified. Decreasing the flow rate to 25 ml h^{-1} and concomitantly using a smaller fraction size of 1.8 ml, significantly improved separation performance. An aliquot of each fraction was analysed for SH content using Ellman's reagent [18]. The fractions containing the individual complexes were pooled, lyophilized, desalted over Sephadex G25 and then subjected to the same procedure again. This gave mg

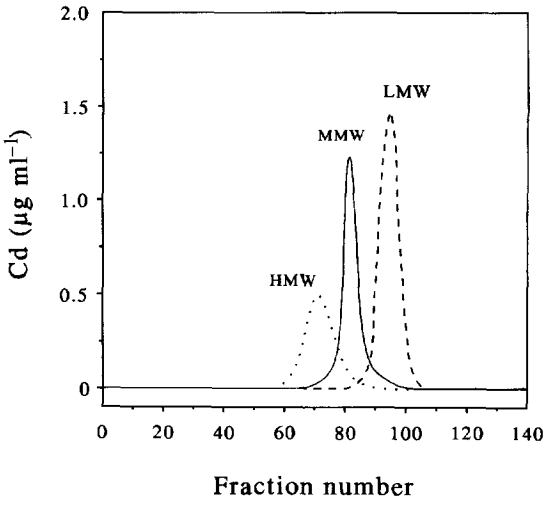

Fig. 1. Re-chromatography of the purified Cd–PC complexes from *Rauvolfia serpentina* on Sephadex G50 (25 × 430 mm, eluent: 10 mM Tris–HCl buffer, pH 8.3, flow rate: 25 ml hr^{-1}). Aliquots of the isolates were applied as follows: 0.4 mg high molecular weight (HMW) complex, 0.2 mg high molecular weight (MMW) complex, and 1 mg low molecular weight (LMW) complex, respectively. The Cd content of the collected fractions (1.8 ml) was determined using atom absorption spectroscopy.

amounts of three distinct Cd–PC complexes which had purified to apparent homogeneity (Fig. 1). As suggested by Abrahamson and coworkers [19], these complexes were designated according to their apparent MW as HMW, MMW (medium-MW), and LMW (low-MW) complexes. The HMW complex was also purified to apparent homogeneity from a Cd–PC isolate of Cd-treated *S. cucubalus* suspension cultures, but it was impossible to separate LMW and MMW in that case.

The PC composition of each of the purified complexes (solubilized in H_2O) was analysed by HPLC with post-column derivatization according to Grill *et al.* [20] and atomic absorption spectroscopy (AAS) for Cd content. Furthermore, a colorimetric S^{2-}-test using the methylene blue method of King and Morris [21] was used. This method proved to be insensitive towards Cd-interference. The results of these analyses are summarized in Table 1.

The analysed isolates differed significantly. The LMW complex from *R. serpentina* consisted mainly of PC_3 and Cd, exhibiting a S:Cd ratio of 3:1. This is the normal S:Cd ratio of Cd–metallothionein and would equate to a Cd^{2+} content of about 13 wt% compared with about 11 wt% found in mammalian Cd–MT [22]. No significant amount of S^{2-} was found in this complex or in the MMW complex from the same plant. For the MMW complex, a S:Cd ratio of 2.4:1 was determined which equates to a Cd content of 16 wt%, thus being about 50% higher than in mammalian Cd–MT. Compared with the LMW complex, the PC chainlength in the MMW complex was less homogenous and significantly longer (Table 1). A significant amount of acid-labile sulphur was detected only in the HMW complex, accounting for 25% of the total sulphur. The S:Cd ratio of 1.9:1 was lower compared with the lighter complexes. Cd was present at 23 wt%, double the amount of the LMW complex or mammalian Cd–MT.

The HMW complex of *Silene* showed an even lower S:Cd ratio of 1.5:1 with a Cd content of 29wt%. Of the total sulphur tested, 30% were sulphide ions while the rest was found predominantely in PC_3 and PC_4 (Table 1).

These results clearly show that Cd complexation by PCs is far more economic than by MTs, especially with regard to the plant growth-limiting elements of sulphur and nitrogen. In mammalian Cd–MT, a minimum S:Cd ratio of 3:1 can be achieved, leading to about 11 wt% of Cd in the complex [22]. In contrast, plants can

Table 1. Composition of the isolated Cd–PC complexes from *Rauvolfia serpentina* and *Silene cucubalus*. All amounts are the actual contents found in 1 mg isolate. PCs (nmol SH per mg isolate) are quantified as γ-Glu-Cys units. The S:Cd ratios represent the actual calculated values, whereas the PC–SH:S^{2-}:Cd ratios were rounded to integer values to represent the natural ratios in the complexes

	R. serpentina			*S. cucubalus*
	LMW	MMW	HMW	HMW
PC_2 (nmol SH)	42	19	14	14
PC_3 (nmol SH)	779	85	246	1069
PC_4 (nmol SH)	71	1064	475	492
PC_5 (nmol SH)	0	317	81	24
PC_6 (nmol SH)	0	238	58	0
PC_7 (nmol SH)	0	108	27	0
PC_n (nmol SH)	892	1832	900	1598
S^{2-} (nmol)	10	18	255	710
Cd^{2+} (nmol)	302	786	624	1574
S:Cd ratio	3:1	2.4:1	1.9:1	1.5:1
PC–SH:S^{2-}:Cd ratio	3:0:1	5:0:2	3:1:2	2:1:2
wt% Cd	13	16	23	29

LMW, low molecular weight; MMW, medium molecular weight; HMW, high molecular weight.

decrease this ratio to 1.5 : 1 and possibly even lower, by incorporating sulphide into the PC complexes. This means a doubled complexation efficiency per sulphur atom and, assuming a Cd content of 30 wt%, requires only one-third occupancy of the ligands.

In order to monitor the kinetics of sulphide and PC formation and to further investigate the role of sulphide incorporation, suspension cultures of *R. serpentina* and *S. cucubalus* were treated with 200 μM $Cd(NO_3)_2$ and analysed for their sulphide and PC contents. While sulphide could not be detected in significant amounts in *Rauvolfia* cells, the sulphide content in *Silene* cells increased dramatically during the period investigated (Fig. 2). Two days after Cd-administration the sulphide content had increased four-fold compared with the control. Maximum sulphide content was reached 7 days after Cd-administration with a 13-fold increase over the control. As expected, PC synthesis was found in both cultures after Cd-administration, but in *Silene* PC levels

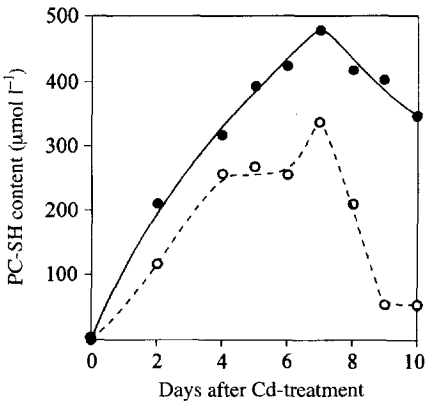

Fig. 3. PC induction in suspension cultures of *Rauvolfia serpentina* (●) and *Silene cucubalus* (○) after treatment with 200 μM $Cd(NO_3)_2$. Note the decrease in PC concentration starting at day 7 which is significantly more pronounced in *S. cucubalus* cultures.

were only two-thirds of that in *Rauvolfia*. In *Silene* cells a drastic decrease in PC levels was observed after 7 days; this was less pronounced in *Rauvolfia* cells (Fig. 3). In order to learn about the role of sulphide incorporation and the drastic PC decrease in *Silene* cultures, the molar ratio PC–SH to sulphide was calculated for each sampling day (Fig. 4). The data shown in Fig. 4 explain why this culture is able to decrease the PC levels so drastically. The decrease in molar PC–SH : sulphide ratio from 30 to 6 indicates that sulphide ions are incorporated increasingly into PC complexes during the observation period. Thereby, the complexation efficiency per sulphur is drastically increased from 3 to 1.5 S per Cd atom, thus allowing the cells to reuse urgently needed amino acids for primary metabolism and protein synthesis without releasing toxic Cd ions. However, the less pronounced decrease of PCs in the *Rauvolfia* culture cannot be explained by sulphide incorporation. Therefore, for both cultures the average

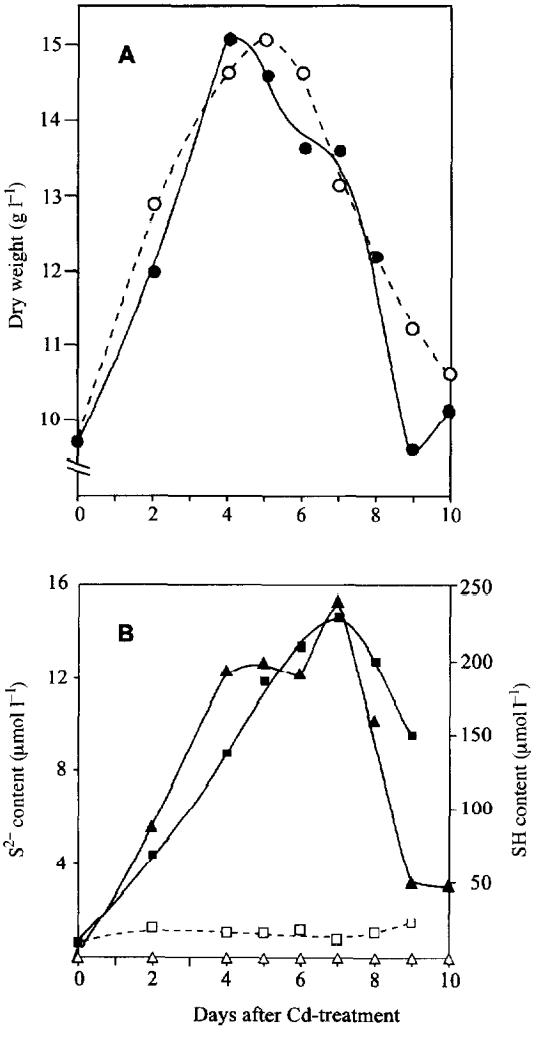

Fig. 2. (A) Dry weight (● control, ○ Cd-treated) and (B) PC content (△ control, ▲ Cd-treated) and sulphide content (□ control, ■ Cd-treated) in *Silene cucubalus* suspension cultures with and without addition of 200 μM $Cd(NO_3)_2$.

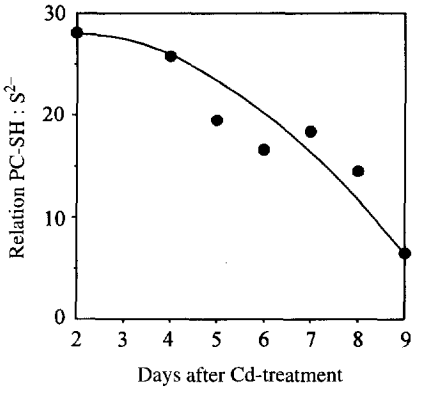

Fig. 4. The PC–SH:S^{2-} ratio in suspension cells of *S. cucubalus* at different times after treatment with 200 μM $Cd(NO_3)_2$. PC concentrations were determined using reversed-phase HPLC with postcolumn derivatization by Ellman's reagent. Sulphide was determined according to ref. [21].

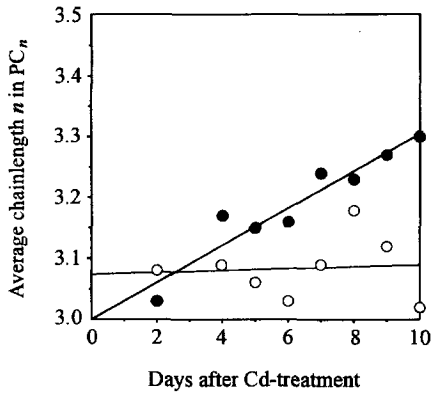

Fig. 5. Average chainlengths (n) of PC_n in suspension cultures of *Rauvolfia serpentina* (●) and *Silene cucubalus* (○) after treatment with 200 μM $Cd(NO_3)_2$. Both data series differ in a statistically significant manner ($\alpha = 0.01$, $P = 0.0074$ for unpaired t-test). Calculated functions for n are $0.031\,x + 3.00$ for *R. serpentina* and $0.002\,x + 3.07$ for *S. cucubalus* where x is the number of days after Cd treatment. These calculations indicate a 15-fold higher trend towards PC chain-elongation in *R. serpentina* cultures.

PC chainlength per sampling day was calculated and the data subjected to statistical analysis assuming a linear trend. Both data series were significantly different ($\alpha = 0.01$) based on a t-test ($P = 0.0074$ for unpaired t-test). As shown in Fig. 5, the average PC chainlength of *Silene* culture was constant at 3.07 during the observation period. In the *Rauvolfia* culture, average chainlength increased from 3 (as in the LMW complex) to 3.3. Due to their obvious inability to synthesize larger amounts of sulphide, *Rauvolfia* cells possibly increase the chainlength of PCs. By forming increasing amounts of MMW complex with higher complexation efficiency per sulphur atom the cells are able to 'recycle' PCs. The observed smaller PC decrease in *Rauvolfia* cultures is in accordance with the higher S:Cd ratio in the MMW complex compared with the HMW complex of *Silene*. Theoretically, in *Rauvolfia* cultures a maximum decrease of the S:Cd ratio from 3 (LMW) to 2.4 (MMW) is possible, while in *Silene* cultures this ratio may decrease by 50% from 3 (LMW) to 1.5 (HMW). In both cases, the *in vivo* observations are in quantitative accordance with the theoretically possible range of decrease in S:Cd ratio.

DISCUSSION

All eukaryotic plants and several fungi detoxify intracellular heavy metal ions via sequestration through phytochelatins. These peptides are synthesized by a constitutive, metal-activated enzyme, PC synthase, from glutathione [1]. It has been demonstrated that the vast majority of intracellular heavy metal ions *in vivo* are bound in PC complexes [16]. Metal–PC complexes *in vitro* exhibit drastically reduced toxicity to metal-sensitive enzymes when compared with the free ions [16].

Several authors [23–25] reported that the transport of Cd–PC complexes into the vacuole is mediated through a specific transport protein [23]. Data from Ortiz *et al.* [24] indicate that sulphide incorporation and vacuolar transport may be linked to each other. These authors were able to complement an initially heavy-metal sensitive mutant of *S. pombe*, unable to form HMW complexes, with a gene, referred to as *htm*1, having significant homology with known ATP-binding transport proteins. This finding was confirmed by Salt and Rauser [23] by the characterization of a MgATP-dependent, vanadate-sensitive transport of Cd–PC complexes across the tonoplast membrane. In combination with the data presented in this paper, these findings suggest a multi-step approach to heavy-metal detoxification and storage in plants and fungi. The first response to heavy-metal ion intrusion is the activation of the PC synthase followed by the formation of LMW complexes, containing rather short PCs and thus needing a high sulphur:metal ratio for efficient metal detoxification, comparable to that of MTs. In *R. serpentina* these LMW complexes are modified by increasing the chainlength thus forming MMW complexes with more efficient use of sulphur. It has been demonstrated *in vitro* [26] that the coordination of Pb^{2+} ions by phytochelatins is dependent on the PC chainlength. In the case of PC_3 one Pb^{2+} ion was bound per peptide, whereas in the case of PC_4 two distinct complexes were formed, binding either one or two ions per peptide. This finding would refer to a S:Pb ratio of 3:1 for a $[(PC_3)Pb]$ complex and a minimum S:Pb ratio of 2:1 in the case of $[(PC_4)Pb_2]$.

In *S. cucubalus* cultures, sulphide is incorporated into the LMW complexes, forming HMW complexes and thus increasing sulphur efficiency. It has been assumed [12] that sulphide incorporation can even provide a means of heavy-metal tolerance, since heavy-metal-tolerant and -sensitive ecotypes of *S. cucubalus* differed mostly in the amount of S^{2-} incorporated into metal–PC complexes. This assumption has been confirmed in the case of a highly Cd-resistant strain of *C. glabrata* by Mehra *et al.* [27]. These authors could demonstrate that this resistance was based solely on the ability of this strain to form extremely high levels of PC-coated Cd–S quantum crystallites. It is not yet clear whether sulphide incorporation takes place in the cytoplasm, in the vacuole, or on the way from cytoplasm to vacuole. The findings of Ortiz *et al.* [24], however, favour the last two possibilities. The fact that Cd–PC complexes are processed and optimized for long-term storage, probably leading to the formation of Cd–S crystallites in the vacuole, makes heavy-metal detoxification through PCs far superior and more adapted to plant specific issues than detoxification through MTs could be. Considering these conditions, a detoxification route via plant MTs is even less likely because of simple, economic reasons despite reports

about plant genes having certain homology with mammalian MTs [28, 29].

EXPERIMENTAL

Plant material. Suspension cultures of *R. serpentina* and *S. cucubalus* were provided by our cell-culture laboratory. Cells were grown in the medium of ref. [30] on a gyratory shaker (100 rpm) at 23° in continuous diffuse incandescent light (650 lx).

Isolation of Cd–PC complexes. Crude Cd–PC complexes were isolated from cell cultures of *R. serpentina* and *S. cucubalus* after 4 days growth in medium containing 200 μM $Cd(NO_3)_2$ according to ref. [14]. A crude Cd–PC prepn (5 mg) was then applied to a Sephadex G50 column (25 × 430 mm) equilibrated with 10 mM Tris–HCl buffer (pH 8.6) containing 50 mM KCl. The sample was eluted with the same buffer at a flow rate of 25 ml hr^{-1}. Frs (1.8 ml) were collected and an aliquot (50 μl) assayed for free thiols using Ellman's reagent (DTNB) [18]. Fractions from each peak were pooled according to the SH elution profile, lyophilized and desalted over a Sephadex G25 column (20 × 350 mm), flow rate: 40 ml hr^{-1}, eluent: H_2O, adjusted to pH 9 using NH_4OH. Each sepd complex was re-chromatographed at least once under the same conditions in order to obtain the desired purity.

Phytochelatin analysis. PC concentrations were determined using RP-HPLC and subsequent postcolumn derivatization of the eluate with DTNB according to ref. [20]. In order to increase the sensitivity of the method, the 5 ml reaction loop for the derivatization reaction was heated to 50° in a water-bath. Purified complexes were dissolved in H_2O prior to analysis, whereas suspension cells were extracted in 0.6 M NaOH containing 1 mg ml^{-1} $NaBH_4$ according to ref. [31].

Sulphide determination. Sulphide ions were determined using the methylene blue method of ref. [21]. 250 μl of a 2.6% aq. soln of Zn-Ac were mixed with 50 μl of a 6% aq. NaOH soln and 350 μl sample for 1 min. Then, 250 μl of a 0.1% soln of *N,N*-dimethylphenylenediamine in 5 MHCl were added and mixed for another 1 min. After the addition of 50 μl of a 11.5 mM $FeCl_3$ soln in 0.1 MHCl, 650 μl of H_2O were added after an incubation time of 30 min at room temp. The *A* of this soln at 695 nm was determined and quantified using a freshly prepared Na_2S soln as a standard. In case of the purified extracts, an aq. soln was used. Whole cells were shock-frozen in liquid N_2 and extracted in 10 mM Tris–HCl buffer (pH 8.3). After removal of cellular debris by centrifugation (10 min, 10 000 *g*, at 4°) the clear supernatant was subjected to analysis.

Cd determination. Cd ions were analysed using AAS in flame mode (acetylene/air) on a Perkin-Elmer PE 1100 B device. Detection wavelength 228 nm; detection limit 0.03 μg ml^{-1}. All data were averaged from triplicate measurements leading, generally, to an S.D. < 1 %.

Thiol determination. SH-groups in frs were assayed using DTNB [18]. Aliquots of each fr. (50 μl) were mixed with 200 μl of a 1 mM DTNB solution in 0.25 M KPi buffer (pH 8) containing 5 mM EDTA in microtitre plate wells. After 5 min reaction at room temp. the absorption was measured at 410 nm using a Dynatech Microplate reader. A fresh, aq. soln of glutathione was used for calibration.

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