PERGAMON

Phytochemistry 50 (1999) 1323–1328

PHYTOCHEMISTRY

Phytochelatins and heavy metal tolerance

I. Leopold [a], D. Günther [b], J. Schmidt [c], D. Neumann [c,*]

[a] *Laboratory for Materials and Nuclear Processes, Paul Scherrer Institute, 5232 Villigen PSI, Switzerland*
[b] *Institute of Isotope Geology and Mineral Resources, Swiss Institute of Technology Zürich, Sonneggstrasse 5, 8092 Zürich, Switzerland*
[c] *Institute of Plant Biochemistry, Weinberg 3, 06120 Halle/S., Germany*

Received in revised form 28 April 1998

Abstract

The induction and heavy metal binding properties of phytochelatins in heavy metal tolerant (*Silene vulgaris*) and sensitive (tomato) cell cultures, in water cultures of these plants and in *Silene vulgaris* grown on a medieval copper mining dump were investigated. Application of heavy metals to cell suspension cultures and whole plants of *Silene vulgaris* and tomato induces the formation of heavy metal–phytochelatin-complexes with Cu and Cd and the binding of Zn and Pb to lower molecular weight substances. The binding of heavy metal ions to phytochelatins seems to play only a transient role in the heavy metal detoxification, because the Cd- and Cu-complexes disappear in the roots of water cultures of *Silene vulgaris* between 7 and 14 days after heavy metal exposition. Free heavy metal ions were not detectable in the extracts of all investigated plants and cell cultures. *Silene vulgaris* plants grown under natural conditions on a mining dump synthesize low molecular weight heavy metal binding compounds only and show no complexation of heavy metal ions to phytochelatins. The induction of phytochelatins is a general answer of higher plants to heavy metal exposition, but only some of the heavy metal ions are able to form stable complexes with phytochelatins. The investigation of tolerant plants from the copper mining dump shows that phytochelatins are not responsible for the development of the heavy metal tolerant phenotypes. © 1999 Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Silene vulgaris*; Caryophyllaceae; *Lycopersicon peruvianum*; Solanaceae; HPLC/ICP-MS; Heavy metal; Phytochelatin

1. Introduction

One of the most important mechanisms in the heavy metal (HM) tolerance of higher plants is the prevention of toxic HM-concentrations in the cytoplasm and organelles. In tolerant plants HMs are very often chelated or precipitated inside the vacuoles (Neumann, zur Nieden, Lichtenberger & Leopold, 1995), indicating a transport of HMs through the cytoplasm. Organic acids such as citrate, malonate or malate are discussed as ligands for HM chelation during their translocation (Brooks, Morrison, Reeves, Dudleey & Akmen, 1979; Brooks, Shaw & Asensi Mafil, 1981; Pancaro, Pelosi, Vernano Gambi, Galoppini, 1978; Mathys, 1977). Due to their low association constant for complex formation (Smith & Martell, 1989) and because high concentrations of these ligands are present in many plants independent from HM tolerance, it is rather unlikely that these organic compounds are involved in tolerance mechanisms. For the transport of Ni an increase of free histidine and the chelating of the HM ions by the imidazole nitrogen of the amino acid was found (Krämer, Cotter-Howells, Charnock, Baker & Smith, 1996). In many cases HM detoxification in plants is linked to the synthesis of cysteine-rich polypeptides, so called phytochelatins (PCs) with the general structure $(\gamma\text{-Glu–Cys})_n$–Gly with $n = 2$–11 (Grill, Winnacker & Zenk, 1985). These compounds are able to chelate HM ions as Cd^{2+} and Cu^{2+} (Howden,

* Author to whom correspondence should be sent.

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PII: S0031-9422(98)00347-1

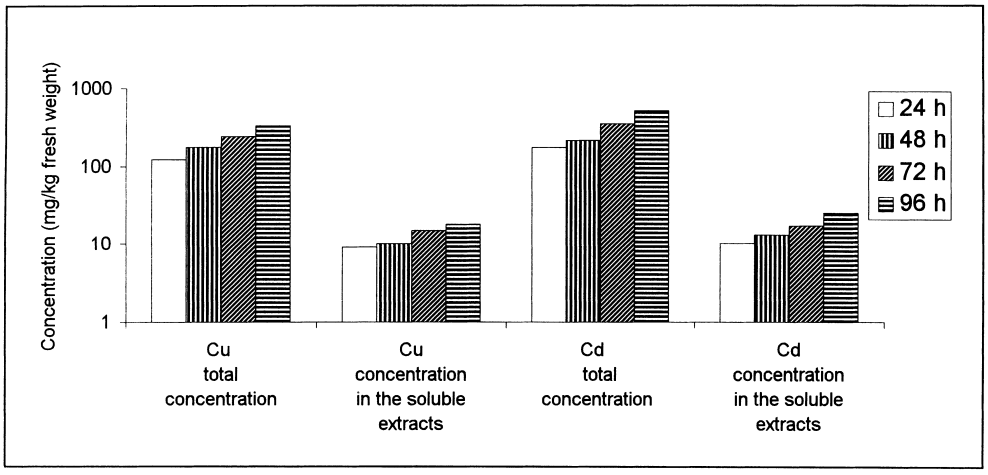

Fig. 1. Time depending Cu and Cd concentrations in cell suspension cultures of *Silene vulgaris* and in the soluble extracts after treatment with 0.1 mM Cu^{2+} and Cd^{2+}.

Goldsbrough, Andersen & Cobbett, 1995; Robinson, Tommey, Kuske & Jackson, 1993), but rather by precipitation of the HMs inside the vacuoles (Lichtenberger & Neumann, 1997). The function of PCs in preventing toxic effects of HMs during the transport across the cytoplasm is controversially discussed, very often the synthesis of PCs does not correlate with HM tolerance (de Knecht, et al., 1994; Delhaize, Jackson, Lujan & Robinson, 1989; Schat & Kolff, 1992).

This work was carried out to investigate soluble HM complexes in sensitive and tolerant plant cells in order to characterize the role of PCs for the development of HM tolerance. Extracts of cell suspension cultures and plants were analysed using an online coupled HPLC/ICP-MS system, which allows the chromatographic separation of different molecules and the quantitative determination of elements bound to the separated compounds in one analytical procedure.

2. Results

2.1. Cell suspension cultures

The time dependent HM accumulation by *Silene vulgaris* cell cultures, incubated with 0.1 mM Cu^{2+} and Cd^{2+} is summarized in Fig. 1. Only about 5% of the total HM content of the cells is present in the soluble extract under conditions used in these experiments. When analysing the soluble extracts of the *Silene vulgaris* cell cultures Cu and Cd elute from the HPLC column within a compound with a molecular weight of approximately 13000 Da (retention time: 8.5 min), which is not detectable in extracts of control cell cultures. For Cd an additional peak with a lower molecular weight was detected within the first 24 h of Cd-treatment. This peak disappears with increased exposition time (Leopold & Günther, 1997). The uptake of the HM ions into the cells and the formation of HM-

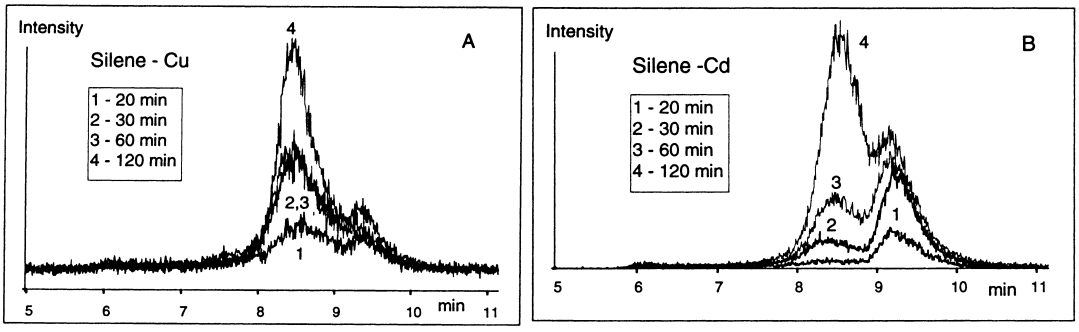

Fig. 2. ICP-MS signals of the soluble extract from a *Silene vulgaris* cell suspension culture after short term incubation with 0.1 mM Cu^{2+} and Cd^{2+}. The time-resolved signals indicate the uptake of Cu (A) and Cd (B) depending on the exposure time. The peaks with a retention time of approximately 8.5 min correspond to the HM–PC-complex.

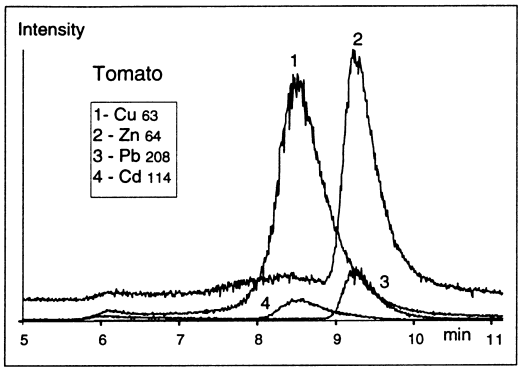

Fig. 3. ICP-MS signals of the soluble extract from a tomato cell suspension culture incubated with 0.01 mM Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} for 5 h. The peak with a retention time of approximately 8.5 min corresponds to the HM–PC-complex. All isotopes of the elements Cu, Cd, Zn and Pb were measured in the HPLC-separated soluble extracts. For the presentation the isotopes with the highest relative abundance are used.

complexes start immediately after HM exposure. Between 20 and 30 min after HM application Cu and Cd can already be detected in the extracts in a complexed form (Fig. 2). In tomato cell cultures similar data were obtained (data not shown). Free HM ions were not found in the investigated cell cultures and plants exposed to different HM ions and concentrations, indicating that metal ions are transported through the cytoplasm in a chelated form. Chelating of free HM ions during the preparation can be excluded by in *vitro* saturation experiments. HMs added during the preparation can be detected as free ions in the soluble extracts.

In tomato and *Silene vulgaris* cell cultures, exposed with equivalent concentrations of Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+}, only Cu- and Cd-complexes, eluting from the HPLC column with a retention time of 8.5 min, were measured, while Pb and Zn are bound to a lower molecular weight ligand (tomato: Fig. 3; for *Silene* see (Leopold & Günther, 1997).

An online coupled HPLC/ICP-MS system for elemental analysis allows the characterisation of the HM ions bound to the HPLC separated components, without any information about the structure or the properties of the HM binding ligands. Therefore, ion electrospray mass spectrometry (ES-MS) was used for the determination of the molecular weight of the ligand in the HM-binding complex. HPLC-separated, purified and concentrated Cu- and Cd-binding complexes with a molecular weight of approximately 13000 Da from cell suspension cultures of *Silene vulgaris* and tomato exposed to Cu^{2+} and Cd^{2+} were dissociated by acidification and analysed by ES-MS. In the samples of both cell cultures the $(M + H)^+$-peak at m/z 538 corresponds to the phytochelatin molecule PC_2 [(γ-Glu–Cys)$_2$–Gly] with oxidised SH-groups. Additional $(M + H)^+$-ions at m/z 479 and 481 corre-

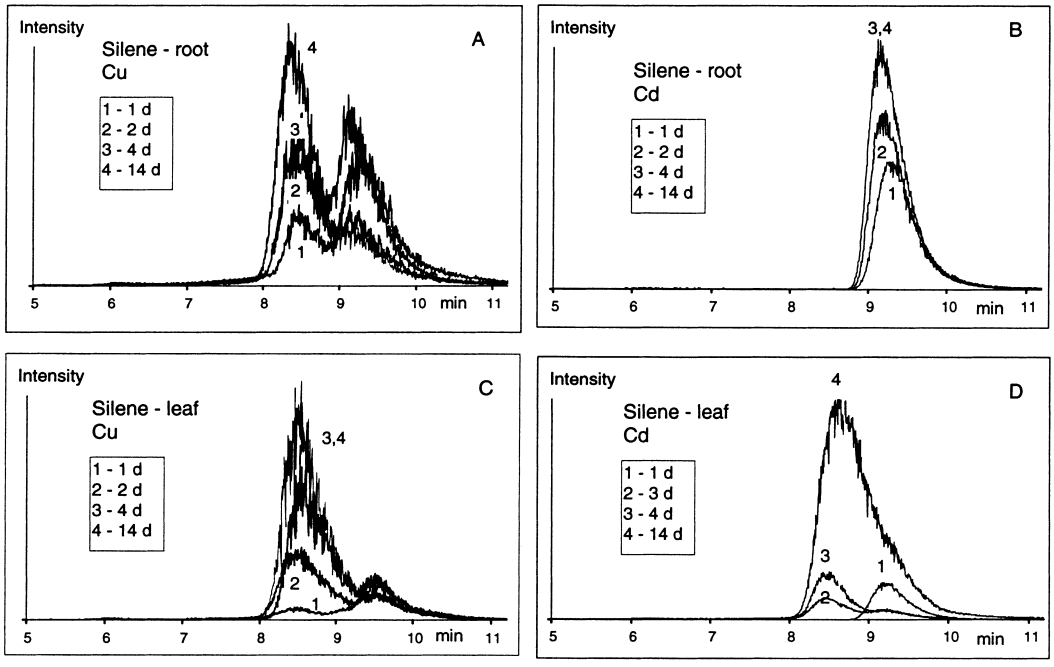

Fig. 4. ICP-MS signals of the soluble extract from water cultures of *Silene vulgaris* after incubation with 0.5 mM Cu^{2+} and Cd^{2+}. The time-resolved signals indicate the uptake of Cu [(A) and (C)] and Cd [(B) and (D)] into roots [(A) and (B)] and leaves [(C) and (D)] of the plants. The peaks with a retention time of approximately 8.5 min correspond to the HM–PC-complex.

spond to the desGly-PC_2 [(γ-Glu–Cys)$_2$] and its reduced form, respectively. The lower molecular ligands measured after Pb and Zn exposure could not be sufficiently purified for mass spectrometry.

2.2. Water cultures

After treatment of *Silene vulgaris* plants in water cultures with different concentrations of Cu^{2+}, Cd^{2+}, Pb^{2+} and Zn^{2+}, significant differences in the HM binding behaviour were observed between the roots and leaves of the investigated plants (Fig. 4). After incubation with 0.5 mM Cu^{2+} and Cd^{2+} a PC-complex was induced in the leaves of *Silene vulgaris* plants [Fig. 4(C) and (D)]. However, in roots only Cu is bound to PC molecules [Fig. 4(A)], whereas Cd forms complexes with the lower molecular weight ligand [Fig. 4(B)]. In *Silene vulgaris* plants, exposed with 0.1 mM Cu^{2+} and Cd^{2+}, a PC-complex with these HM ions was found as well in roots as in leaves of these plants. The investigation of the long-term binding properties of PC-complexes in *Silene vulgaris* showed that especially at concentrations of 0.1 mM Cu^{2+} and Cd^{2+} the amounts of the HM–PC-complexes reach a maximum already between 2 and 4 days. The Cu- and Cd–PC-complexes disappear in the extracts between 7 and 14 days after HM incubation and both ions are bound to the lower molecular weight compound (data not shown), which explains that the synthesis of PC molecules and the binding of HM ions to these peptides seem to be only a transient process in whole plants. This behaviour was not observed in cell suspension cultures of the same plant incubated with equivalent HM concentrations.

The soluble extracts from tomato plants incubated with different concentrations of Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} show a very similar induction and binding characteristic as *Silene vulgaris* plants (data not shown).

In accordance to the cell culture system the whole plants of tomato and *Silene vulgaris* do not form complexes of Zn or Pb ions with PC molecules. Zn and Pb are bound to lower molecular weight ligands with the same retention time observed in the cell suspension cultures of *Silene vulgaris* and tomato.

2.3. Tolerant plants from a natural habitat

Silene vulgaris grown on a medieval copper mining dump and two additional plant species from this ecosystem (*Armeria maritima, Minuartia verna*) were extracted in the same way as the cell cultures and the soluble fraction was separated and analysed by HPLC/ICP-MS. In root and leaf samples of the three different plant species no HM–PC-complexes were detected. The metal ions are always chelated by lower molecular weight ligands (Fig. 5). The investigation of the plants from the copper mining dump using a reversed-phase chromatographic assay with post-column derivatization for the analysis of SH-containing compounds confirm that PCs are not present in the soluble extracts of these plants (Bringezu, personal communication).

3. Discussion

To test the principal reaction of higher plant cells to HM-stress tolerant (*Silene vulgaris*) and sensitive (tomato) cell suspension cultures are used as a simplified model system. *Silene vulgaris* cells tolerate 5 to 10 times higher concentrations of Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} in comparison to tomato cell cultures. From both cell lines soluble HM-complexes were isolated. After Cu and Cd exposure the ligands of the com-

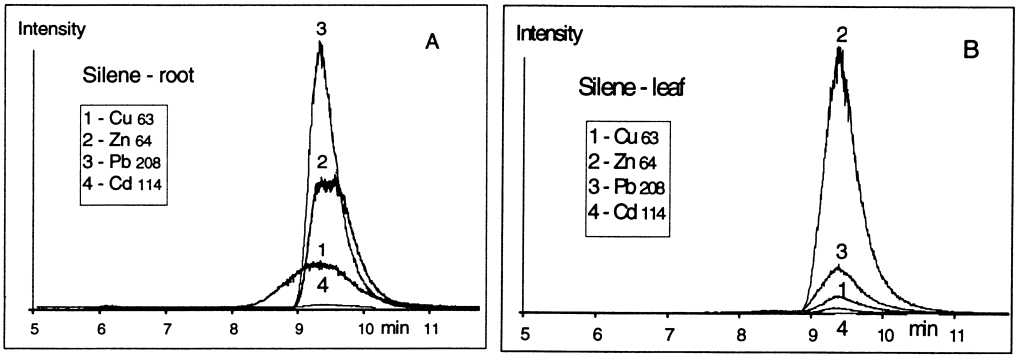

Fig. 5. ICP-MS signals of the soluble extract from *Silene vulgaris* plants grown on a medieval copper mining dump. The time-resolved signals indicate the HM uptake into roots (A) and leaves (B). All isotopes of the elements Cu, Cd, Zn and Pb were measured in the HPLC-separated soluble extracts. For the presentation the isotopes with the highest relative abundance are used.

plexes are identified by ES-MS as a mixture of PC_n and desGly-PC_n isoforms with $n = 2$. Because free SH-groups are necessary for the metal chelation in the PCs the S–S-bridge observed in the mass spectrum seems to be the result of the ionisation process in the mass spectrometer. Such rearrangement reactions in PC-complexes were observed also by (Meuwly, Thibault, Schwan & Rauser, 1995) and (Salt & Rauser, 1995). The binding of HM ions to PC molecules starts very quickly in both cell lines. Already 20 to 30 min after Cu and Cd application HM–PC-complexes can be detected in tomato and *Silene vulgaris* cell cultures. PC isoforms larger than $n = 2$ are not involved in the binding of HM ions during the first 24 h after HM exposure in the investigated concentration range.

A comparison with data from *Schizosaccharomyces pombe, Saccharomyces cerevisiae* and *Zea mays* indicates that the desGly-PC isoforms, found in the isolated complexes, are native in *Silene vulgaris* and tomato and are not a result of the ionisation process in mass spectrometry (Leopold & Günther, 1997; Meuwly *et al*., 1995; Kneer, Kutchan, Hochberger & Zenk, 1992; Kon-Ya, Yoshimura, Yamazaki & Toda, 1990). In *Silene vulgaris* and tomato cell cultures a mixture of PCs and desGly-PCs is responsible for the detoxification of Cu^{2+} and Cd^{2+} during the transport through the cytoplasm. Additionally, at least for Cd the PCs are participated in the formation of CdS/PC-precipitates inside the vacuoles of tomato cell cultures (Lichtenberger & Neumann, 1997).

In contrast to Cu and Cd, Pb and Zn are chelated by lower molecular weight ligands, probably organic acids, as suggested by (Mathys, 1977) or SH-containing compounds, as indicated by the comparison of the retention time and binding behaviour of cysteine and glutathione complexes (data not shown). Although Pb and Zn induce the formation of PCs (Maitani, Kubota, Sato & Yamada, 1996), they are not able to form complexes with PCs, which may be due to the different mechanisms of complex formation between PC molecules and these HM ions. Whereas Cd^{2+} binds to the SH-group of the cysteine residue, Cu^{2+} and other ions of 3d-transition metals are chelated by free electron pairs of the N- and O-atoms of the peptide bound (Krämer *et al*., 1996; Lichtenberger & Neumann, 1997). In addition, a variety of in *vivo* and in *vitro* HM saturation experiments in *Silene vulgaris* cell cultures verified the higher binding stability of Cu^{2+} ions to the PC-complex in comparison to Cd^{2+} ions (Leopold and Günther, 1997). Pb^{2+} and Zn^{2+} are unable to form electronic or thermodynamic stable complexes with PCs. Unfortunately, the Pb- and Zn-complexes, eluting later than PCs from the HPLC column, could not be sufficiently purified for mass spectrometry and could not be characterised completely.

Cell suspension cultures admit certainly a principle mechanism of a plant cell to respond to a stress situation. However, differentiation processes can alter the behaviour of the cells and cell suspension cultures are not comparable in all details with whole plants. This is supported by the data obtained with Cu- and Cd-treated plants of *Silene vulgaris* and tomato. Whereas in roots only Cu was bound to PCs depending on the concentration, in leaves both metal ions form PC-complexes. However, this is a transient effect only. With prolonged time of the HM exposure the PC complexes can disappear and the metals are chelated by lower molecular weight ligands. For extracts of HM-tolerant plants of *Silene vulgaris, Armeria maritima* and *Minuartia verna*, grown on a medieval copper mining dump, completely different data have been measured. Neither in roots nor in leaves HM–PC-complexes can be found, which may be another indication that the complexation of HM ions by PCs is only a transient process.

In principle, tolerant and sensitive cells are able to synthesise PCs under HM-stress. Undifferentiated cell suspension cultures as well as whole plants in water cultures show the same binding properties: Cu and Cd ions are mainly complexed by PC molecules, whereas Zn and Pb ions are bound to an up to now uncharacterised low molecular weight compound. The synthesis of HM–PC-complexes is a fast and probably transient answer of the cells and not necessarily important for the HM tolerance of plants, which are able to grow on HM-polluted soils under natural conditions.

4. Experimental

4.1. Plant material

Cell suspension cultures of *Silene vulgaris* (Moench) Garcke and *Lycopersicon peruvianum* L. were cultivated according to (Murashige & Skoog, 1997). For stress experiments the cells were used after 4 days (*Silene vulgaris*), respectively 1.5 days (tomato) at the end of the logarithmic growth phase. Cell suspension cultures of *Silene vulgaris* were incubated with 0.01 to 0.1 mM of Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+} for up to 2 weeks. The same conditions for tomato cell cultures with 10 times lower concentrated solutions were used. The time interval between each individual sampling was varied between 10 min for the investigation of the

short term accumulation and 1 week for the characterisation of the long term HM uptake.

Plants of *Silene vulgaris* (Moench) Garcke, ssp. humilis (Schubert) Rauschert were collected from a medieval copper mining dump (''Saugrund''/Eisleben, Germany). Seeds from the same source were used for the water cultures. The plants were grown under controlled greenhouse conditions (16 h light/8 h darkness, 24°C/20°C, 55% relative humidity) for 4 weeks in Zindzadse solution. The *Silene vulgaris* plants were incubated with 0.1 to 0.5 mM Cu^{2+}, Cd^{2+}, Zn^{2+} and Pb^{2+}; a 10 times lower concentration was used for tomato plants.

4.2. Extraction of the plant material

Cell suspension cultures were harvested by suction filtration, washed with 0.1 M EDTA to remove extracellulary bound HM ions, resuspended twice with deionized water and filtered again. The same wash procedure was applied to the roots of all investigated plants. The sample preparation of the soluble extracts of cell suspension cultures and whole plants was carried out as described before (Leopold & Günther, 1997).

4.3. HPLC/ICP-MS

The HPLC system (LKB, Bromma, Sweden) was equipped with two chromatographic pumps, an LC controller, a variable wavelength monitor and an integrator. Eurogel GFC-OH and Eurogel GFC, both with a particle diameter of 8 μm, were used as guard and analytical column for size exclusion chromatography. Samples were applied to the chromatographic system using a Rheodyne injection valve (equipped with a 20 μl loop) and eluted with 10 mM ammonium acetate buffer (pH 7) at a flow rate of 1.0 ml/min. Calibration standards for the ICP-MS were injected into the mobile phase using a second Rheodyne injection valve that was installed downstream from the variable wavelength monitor.

Polyfluoralkoxy tubings were used for the connection between the outlet of the second injection valve and the nebulizer of the ICP-MS system. The ICP-MS instrument (PQ2 + , VG Elemental, Winsford, U.K.) was equipped with a water-cooled Scott-type spray chamber and a V-groove nebulizer. The detailed operating parameters of the online coupled HPLC/ICP-MS system are described earlier (Leopold & Günther, 1997).

The amounts of Cd and Cu selectively bound to ligands in the soluble extracts were quantified from the HPLC/ICP-MS experiments using HM–EDTA-complexes as calibration standards. The total HM concentrations of the cell cultures were determined by direct nebulization into the ICP-MS after digestion in a microwave digestion system (MDS 205, CEM, Matthews, U.S.A.).

4.4. Electrospray mass spectrometry

The positive ion electrospray (ES) mass spectra were obtained from a Finnigan TSQ 7000 instrument (electrospray voltage 4.5 kV, sheath and auxillary gas: N_2, syringe pump).

Acknowledgements

We thank Dr O. Lichtenberger for the quantum-chemical calculations of the binding behaviour between heavy metal ions and phytochelatin molecules.

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