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Characterization of cadmium- and lead- phytochelatin complexes formed in a marine microalga in response to metal exposure

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Abstract

Phytochelatins (PC_n) are thiol-containing peptides with general structure (γ-Glu-Cys)$_n$-Gly enzymatically synthesized by plants and algae in response to metal exposure. They are involved in the cellular detoxification mechanism for their capability to form stable metal-phytochelatin complexes. The speciation of Cd and Pb complexes with phytochelatins has been studied in laboratory cultures of the marine diatom *Phaeodactylum tricornutum*. An approach based on size-exclusion chromatography (SEC) with off-line detection of phytochelatins, by reverse-phase HPLC, and metal ion, by atomic absorption spectrometry, has been used. The formation of Cd- and Pb-PC_n complexes with *n*-value from 3 to 6 was demonstrated. The metal-PC_n complexes formed with Cd appear to be different from those formed with Pb for the number of molecules of peptide involved in the complex and for the amount of the metal ion bound. The chromatographic behaviour of metal-PC_n complexes is consistent with Pb-PC_n complexes in which only a molecule of peptide binds the metal ion, and with Cd-PC_n complexes containing two or more molecules of peptide. The metal/peptide molar ratio in Cd-PC_n complexes was higher that in Pb-PC_n complexes. The formation of Cd- or Pb-PC_2 complexes was not demonstrated, probably for a dissociation during the cellular extract preparation. The effectiveness of phytochelatins in the detoxification of these two metal ions in this alga is discussed.

Introduction

Biological systems respond to potentially toxic levels of metal ions mainly by chelation and sequestration of the excess of the element into harmless compounds. Plants, algae and fungi use intracellular metal-binding peptides, trivially known as phytochelatins, for metal homeostasis and detoxification (Rauser 1995; Zenk 1996). Phytochelatins (PC_n) are a family of peptides, with general structure (γ-Glu-Cys)$_n$-Gly ($n = 2$ to 11), synthesized by the constitutive enzyme phytochelatin synthase, which is activated by the presence of metal ions (Grill *et al*. 1985; Grill *et al*. 1989). Owing to the high cysteine content, phytochelatins bind metal ions forming intracellular metal complexes, thereby reducing the intracellular free metal ion concentration. *In vitro* experiments have shown that phytochelatins are able to protect metal-sensitive enzymes from the inactivation and to restore the activity of metal-poisoned enzymes (Kneer & Zenk 1992).

For some plants it has been shown that not all the species of metal ions capable to induce PC_n are able to form stable metal-PC_n complexes (Leopold & Gunther 1997; Maitani *et al*. 1996). So, it has been underlined (Yen *et al*. 1999) that it is the formation of stable complexes that is important for metal tolerance rather than the ability to synthesize phytochelatins. Thus, the knowledge of the metal-binding characteristics of these bioinduced peptides is a key factor for the understanding of their role in cellular detoxification mechanisms and in heavy metal tolerance.

Although many studies have been reported on the PC_n synthesis by phytoplanktonic algae, they have mainly regarded the relationship between metal dose

and PC_n production, the response to a variety of metals, or interspecies comparisons (Ahner & Morel 1995; Ahner *et al*. 1995, 1997; Knauer *et al*. 1998). Only few papers are appeared in the literature on the characterization of the complexes formed between the metal ion and these peptides in phytoplanktonic algae (Gekeler *et al.* 1988; Morelli & Scarano 1995). Studies on the nature of these complexes have mainly regarded the higher plants (Kneer & Zenk 1997; Hu & Wu 1998; Rauser 2000; Vacchina *et al*. 2000).

The approaches devoted to the isolation of metal-PC_n complexes involve the use of separative techniques, as size exclusion chromatography (SEC) or anionic exchange chromatography, coupled with the detection of the metal-thiolate bond, by the UV signal at 254 nm, and of the metal ion by atomic absorption spectrometry (AAS) or inductively coupled plasma mass spectrometry (ICP-MS). These detection methods don't supply information about the nature of the isolated compounds, whereas techniques with high molecular specificity are required. Reverse-phase high performance liquid chromatography (RP-HPLC) allows to detect, in a single HPLC run, phytochelatins of different chainlength, after acidification of the sample and derivatization of thiols by DTNB (Grill *et al*. 1987) or monobromobimane (Fahey & Newton 1987).

In this work size exclusion chromatography was coupled with RP-HPLC and with AAS to study the binding of Cd and Pb to phytochelatins synthesized by cells of *Phaeodactylum tricornutum* exposed to these metal ions. This alga, an unicellular marine diatom, responds to exposure to Cd or Pb by synthesizing PC_n of different degree of polimerization, with *n*-values from 2 to 6 (Morelli & Scarano 2001). The objectives of this study were the assessment of the distribution of the intracellular metal ions among individual peptides and the characterization of metal-PC_n complexes differing in peptide chainlength.

Materials and methods

Culturing conditions

The unicellular marine diatom *P. tricornutum* Bohlin (Bacillariophyceae) was grown in axenic conditions, at 23 °C, using continuous illumination under fluorescent daylight (100 μmol photons $m^{-2} \times s^{-1}$). The growth medium was natural seawater enriched with f/2 (Guillard 1975) at one fifth the normal trace metal concentration and without Cu. Before f/2 addition, natural seawater was sequentially passed through a bed of charcoal and a Chelex column at the flow rate of 1 ml $\times$ min^{-1}, in order to reduce both dissolved organic matter and trace metals concentration. The culture medium was sterilized by filtration on 0.2 μm sterile membrane filters (Sartorius). Stock cultures were maintained in batch by inoculating every 7 days into a fresh culture medium. Calculated volumes of these stock cultures of *P. tricornutum* were used as inoculum in the metal exposure experiments. One-liter cultures, at an initial cell concentration of 3×10^4 cells $\times$ ml^{-1}, were grown for five days up to the beginning of the stationary growth phase (cell density, $2–3 \times 10^6$ cells $\times$ ml^{-1}), then Cd or Pb were added to obtain a concentration of 10 μM. After 6 h the cells were harvested. These exposure conditions were found to be adequate to avoid toxic effects during metal exposure as well as to induce enough amounts of phytochelatins to allow the detection of their metal complexes. Cell counts were carried out by means of a Thoma counting chamber under a microscope.

Preparation of the cell homogenates

Metal-treated algae were collected by filtration onto 0.8 μm membrane filter (Sartorius), then rinsed extensively with natural seawater. The algae were immediately placed in 2–3 ml 50 mM N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES), 50 mM NaCl pH 7.5 buffer, containing 1 mM Tris (2-carboxyethyl) phosphine (TCEP) as antioxidant, then disrupted by sonication (Sonopuls Ultrasonic Homogenizer, Bandelin) for 10 min with a repeating duty cycle of 0.3 s. All extraction procedures were carried out in an ice bath. The cellular homogenate was then transferred to Eppendorf centrifuge tubes and centrifuged for 15 min at 4 °C to pellet cell fragments. The supernatant was filtered through 0.2 μm membrane filter and immediately submitted to the SEC procedure. An aliquot of the filtrate was used, after proper dilution, for the determination of Cd or Pb, by AAS, and of non-protein thiols, by RP-HPLC.

Size exclusion chromatography

Size exclusion chromatography was performed by using a Hi-Load Superdex 30 preparation grade column (60×1.6 cm; Pharmacia Biotech), designed for the separation of peptides and small proteins, with an effective separation range below 10 kDa. The SEC system consisted of a Pharmacia Biotech Pump P-50,

a Rheodyne model IV-7 injection valve, an UV detector (Uvicord SD, Pharmacia LKB) set at 254 nm. The column was calibrated by using, as molecular weight standards, the following compounds: γ-EC (250 Da), GSH (307 Da), PC_2 (538 Da), PC_3 (770 Da), PC_4 (1002 Da), PC_5 (1234 Da), PC_6 (1466 Da), aprotinin (6500 Da) and cytochrom c (12327 Da).

The elution buffer was 50 mM HEPES, 50 mM NaCl pH 7.5, flowing at 1 ml $\times$ min^{-1}. An aliquot of 1 ml of the cell homogenate was injected in the column and the eluate was collected in 1-ml fractions. Before injecting the next sample, the column was cleaned by loading 3 ml of 0.1 M EDTA. Each collected fraction was analysed for non-protein thiols by HPLC and for Cd or Pb by AAS using a Perkin Elmer Spectrophotometer (Model 1100 B) equipped with a graphite furnace (Model HGA 700).

Determination of non-protein thiols

Non-protein thiols were separated and quantified after derivatization with the fluorescent tag monobromobimane (mBrB) (Newton *et al.* 1981; Fahey & Newton 1987) by following the procedure reported elsewhere (Morelli & Scarano 2001) with some modifications. Briefly, a volume of 100 μl of the sample was acidified by adding 10 μl of 1.2 M HCl/50 mM diethylenetriaminepentacetic acid (DTPA), in order to dissociate the metal-PC_n complexes, and allowed to stand for 30 min. Then, 130 μl of 200 mM 4-(2-hydroxyethyl)-piperazine-1-propane-sulfonic acid (HEPPS)/5 mM DTPA, pH 8.2, and 10 μl of 10 mM TCEP, dissolved in the same buffer, were added to this solution in order to protect the thiol groups from oxidation. After 15 min of incubation, 20 μl of 10 mM mBrB in acetonitrile were added and the reaction was allowed to proceed for 15 min in the dark at 45 °C. Afterwards, 20 μl of 100 mM cysteine were added in order to react the remaining mBrB and, 15 min later, the reaction was stopped by the addition of 20 μl of 1 M methanesulfonic acid (MSA). The derivatized samples were stored in the dark at +4 °C until HPLC analysis. Blank samples were used to identify the reagent peaks.

The bimane derivatives were separated on an Alltech Econosphere C-18 5 μm reverse-phase column (250 mm $\times$ 4.6 mm) and detected at 380 nm excitation and 470 nm emission wavelengths. 0.1% trifluoroacetic acid (TFA) was used as solvent A and 100% acetonitrile as solvent B. A linear gradient from 10% to 30% solvent B, for 50 min, was used. The flow rate was 1 ml $\times$ min^{-1}. Before injecting a new sample, the column was cleaned (5 min, 100% acetonitrile) and allowed to equilibrate (10 min, 10% acetonitrile). All solvents were degassed before the use.

Retention time of phytochelatin oligomers was checked with PC_2, PC_3 and PC_4 *Silene vulgaris* standards (Friederich *et al.* 1998) kindly provided by Prof. M.H. Zenk, Munich University, Germany. Retention time of PC_5 and PC_6 was calculated from the linear relationship between the retention time of the standard peptides and log *n*, where *n* is the number of γ-Glu-Cys dipeptide pairs, as described by Gekeler *et al.* (1989). Phytochelatins were quantified by using the relationship peak area *vs* concentration of GSH standard solutions.

The HPLC system consisted of two Shimadzu LC-10 AD pumps, a Rheodyne 7725 injection valve connected to a 100 μl loop, a RF-10 AXL Shimadzu fluorescence detector. Chromatographic data were processed using Chromatoplus software.

Chemicals

All reagents were analytical grade: mBrB and TCEP were from Molecular Probes; DTPA, GSH, cysteine and HEPES were from Fluka; HEPPS and γ-Glu-Cys (γEC) were from Sigma; MSA, HCl and HNO_3, Suprapur grade, were from Merck; acetonitrile and TFA, HPLC grade, were from Baker; EDTA, $CdCl_2$ and $Pb(NO_3)_2$ were from Carlo Erba. Water was purified by a Milli-Q system (Millipore). TCEP and mBrB were prepared daily, the other solutions were prepared weekly and stored in the dark at +4 °C.

Results

Reverse-phase HPLC assays of non-protein thiols in extracts of cells exposed to Cd or Pb (10 μM Cd or Pb, 6 h) revealed in both the presence of phytochelatins with *n*-value from 2 to 6. The percentage of the individual oligomers (expressed as γ-Glu-Cys units) was 26, 40, 25, 6 and 3%, in the extract from Cd-exposed cells, and 49, 25, 14, 9 and 3% in the extract from Pb-exposed cells, for PC_2, PC_3, PC_4, PC_5 and PC_6, respectively, thus indicating that Cd induced a higher amount of more polymerized peptides than Pb.

Size exclusion chromatography of crude extracts of cells exposed to Cd is shown in Figure 1A. Two main UV absorption peaks, in the elution zone going from fraction 67 to 91, were found. The chromatogram obtained from Cd assays follows in this elution zone

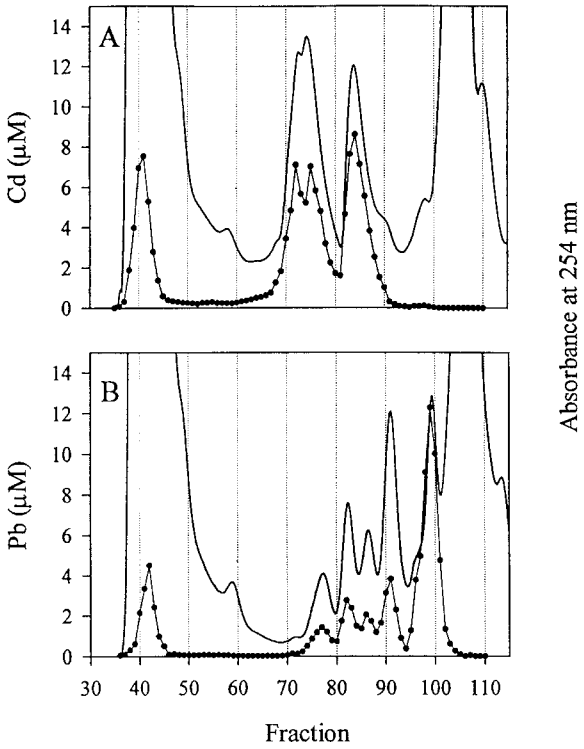

Fig. 1. SEC of crude extracts of 1×10^9 cells of *P. tricornutum* exposed for 6 h to 10 μM Cd (A) or Pb (B). Solid lines: absorbance at 254 nm. SE column: Hi-Load Superdex 30 (60 × 1.6 cm; Pharmacia Biotech). Elution buffer: 50 mM HEPES, 50 mM NaCl, pH 7.5. Flow rate: 1 ml × min^{-1}.

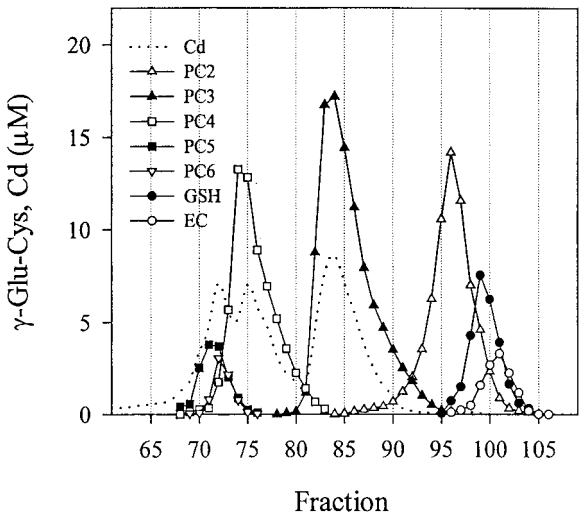

Fig. 2. Concentration of non-protein thiols (γ-Glu-Cys, μM) in the fractions of the SEC reported in Figure 1A. Dotted line refers to the Cd distribution in the SEC eluate.

the UV profile, with the Cd peak at higher MW enlarged and composed by two o more overlapping peaks. These UV and Cd peaks were absent in the chromatogram of the extract of not exposed cells, spiked with Cd (data not shown). Cd bound to the inducible material accounted for over 80% of total Cd in the cellular extract (140 nmol × ml^{-1}), the remaining Cd was eluted at the exclusion volume of the column (fractions 38–45) bound to cellular components of very high MW. Extracts of cells exposed to Pb (Figure 1B) gave several UV absorption peaks in the elution zone going from fraction 73 to 103, matching the Pb chromatogram. The metal recovered in this elution zone accounted for 80% of total Pb in the cellular extract (100 nmol × ml^{-1}), being the remaining Pb associated with HMW cellular compounds.

PC_n chromatograms, obtained from off-line reverse-phase HPLC determination of non-protein thiols on each 1-ml fraction of SEC eluate, are shown in Figure 2 for Cd-exposed cells. A sequential elution of the oligomers, from longer to shorter chains, occurs with increasing elution volume. Cd co-elutes with PC_n with n-values from 3 to 6, indicating the presence of the respective Cd-PC_n complexes in the cellular extract. Cd was absent in the elution zone of PC_2, GSH and γ-EC. Cd-PC_4 eluted well separated from Cd-PC_3 at an earlier elution volume, showing that Cd forms distinct complexes with each oligomer and that these have an apparent molecular weight according to the polimerization degree of the oligopeptide involved. Cd-PC_5 and Cd-PC_6 complexes appear to co-elute in the fractions from 72 to 75, although the Cd-PC_5 complexes begin to elute earlier (fractions 70 and 71). It was possible to obtain a better separation of these peptides by using a higher NaCl concentration (200 mM) in the mobile phase. The results (data not reported) showed that $CdPC_5$ and $CdPC_6$ behave as two distinct complexes and that the former exhibits a slightly higher apparent molecular weight.

Figure 3 shows the PC_n chromatograms of Pb-exposed cells. The distribution of both the oligomers and Pb, in the collected fractions, shows that Pb-PC_n complexes with n-value from 3 to 6 were present in the cellular extract. The Pb peak in the elution zone from fraction 96 to fraction 103 appears between the trailing edge of the PC_2 peak and the leading edge of the GSH peak, showing its maximum at 99 ml. Although a little amount of Pb could be bound to PC_2 (shoulder at 96 ml), the Pb peak doesn't match either PC_2 or GSH peak. So, as in the Cd experiment, the major amount of PC_2 in the cellular extract was collected in the metal-free form. This finding also shows that a large amount of Pb (about 40%) was bound not to PC_n,

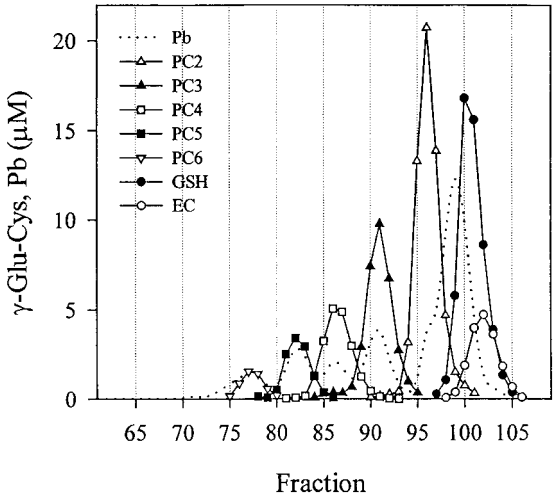

Fig. 3. Concentration of non-protein thiols (γ-Glu-Cys, μM) in the fractions of the SEC reported in Figure 1B. Dotted line refers to the Pb distribution in the SEC eluate.

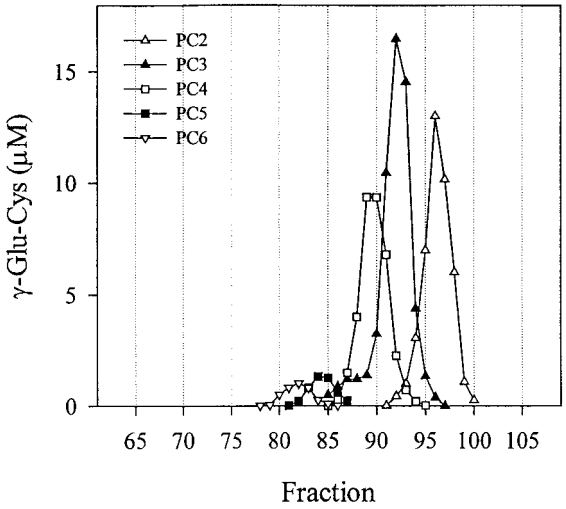

Fig. 4. Chromatograms of apo-PC_n. Metal-free phytochelatins were obtained by treating with DTPA an extract of Cd-exposed cells (see text).

but to unknown LMW compounds, thus indicating that the metal ion bound to synthesized phytochelatins accounts only for 40% of the total dissolved Pb in the cellular extract. A shorter exposure (1 h) to Pb again showed that a significant percentage of the metal was eluted at the LMW zone. Figure 3 also shows that Pb-PC_n complexes were eluted according to the size of the oligopeptide involved in the complex. A comparison with PC_n chromatograms of Cd-exposed cells reveals that, for a given value of n, Pb-PC_n complexes eluted as compounds characterized by an apparent molecular weight lower than Cd-PC_n complexes.

In order to assess whether the amount of PC_n with $n > 2$, detected in each 1-ml fraction, was fully bound to the metal ion, the elution volumes of apo-PC_n were assessed by using a mixture of apo-peptides, obtained by treating with an excess of DTPA an extract of cells exposed to Cd. The distribution of Cd in the SEC eluate indicates that both Cd bound to HMW cellular components as well as Cd originally bound to PC_n, was displaced by DTPA. The chromatogram of the mixture of apo-PC_n (Figure 4) shows the elution volumes of each different peptide. Apo-PC_2 elutes at the same elution volume as found in the chromatograms of the metal-treated cells. The elution volume of the Cd-PC_n complexes is significantly different from that of the respective apo-PC_n, showing that apo-PC_n with $n > 2$ were absent in the cellular extract. Pb-PC_n complexes elute at elution volumes close to the respective apo-PC_n, (1–4 ml earlier). However, whether apo-PC_n were present in the cellular extract, they should produce a shoulder on the trailing edge of Pb-PC_n peaks. These shoulders were not detected in peaks of Figure 3, so, it can be assumed that apo-PC_n with $n > 2$ were absent in this cellular extract.

These findings indicate that all the amount of the oligomers with $n > 2$, assayed in each 1-ml fraction of SEC, was bound to the metal ion. So, an evaluation of the metal/peptide ratio of complexes can be made. For this purpose we replicated 3 times the experiments. By selecting, on the basis of the PC_n chromatograms, the fractions containing only one type of PC_n oligomer, a molar ratio metal/peptide of 1.7 ± 0.2, 2.6 ± 0.3, 1.0 ± 0.2 and 1.6 ± 0.1 for Cd-PC_3, Cd-PC_4, Pb-PC_3 and Pb-PC_4, respectively, was estimated.

An estimate of the apparent molecular weight of Cd- and Pb-PC_n complexes was obtained by calibrating the column with apo-PC_n (Figure 4), cytochrome c, aprotinin, GSH and γ-EC. The apparent MW were 1400, 3000, 4700 and 4500 for Cd-PC_3, Cd-PC_4, Cd-PC_5 and Cd-PC_6, respectively and 800, 1200, 1900 and 2600 for Pb-PC_3, Pb-PC_4, Pb-PC_5 and Pb-PC_6, respectively.

Discussion

Our results show that Cd and Pb are able to induce the PC_n synthesis in *P. tricornutum* and that both elements are capable to form stable complexes with PC_n with $n = 3$–6. As performed in this work, size exclusion chromatography, by using a semi-preparative column,

appears to be adequate to individuate, and to partially separate, the individual families of Cd- and Pb-PC_n complexes accumulated in short term exposure by *P. tricornutum* in its stationary growth phase.

Although an unambiguous identification of the size and stoichiometry of individual metal-PC_n complexes can only be obtained through the use of techniques with high mass resolution (Yen *et al*. 1999), some considerations on the size of metal-PC_n complexes can be drawn from our results.

PC_n form with Pb complexes characterized by an elution volume similar to that of the apo-PC_n, so suggesting that these complexes would contain only one peptide in their molecule. On the contrary, Cd-PC_n complexes eluted at elution volumes much lower than apo-PC_n in agreement with the formation of Cd-PC_n complexes containing at least two peptides in their molecule.

The estimated molar ratios metal/peptide, higher in Cd-PC_n than in Pb-PC_n complexes ($n = 3, 4$), suggest that the same amount of synthesized PC_n is capable to sequester more Cd than Pb ions. So, Cd-PC_n differ from Pb-PC_n for the number of peptides involved in the molecule as well as for the amount of the metal ion bound. Therefore, it could be expected that a given peptide can have a different ability to stabilize these metal ions by forming complexes with a different molecular structure and stoichiometry.

From our results, it can be inferred that the role played by phytochelatins in the sequestering process of these two metal ions is different: (1) over 80% of Cd in cellular extract was detected in a complexed form, but only 40% of cellular Pb was incorporated into phytochelatins, (2) the molar ratio metal/peptide was higher in Cd-PC_n than in Pb-PC_n complexes, and, as a consequence, an equal amount of synthesized PC_n is expected to sequester more Cd than Pb ions, (3) Cd induces a PC_n cellular pool composed by more polymerised oligomers than Pb, and more polymerised PC_n bind a higher amount of metal. These findings seem to suggest that the bio-induction of phytochelatins acts, in this alga, as a detoxification mechanism less effective for Pb than for Cd. It could be that other cellular compounds (organic or inorganic acids) are capable to bind Pb and they could be somehow involved in the detoxification of Pb. Studies based on HPLC-ICP-MS, performed on plants exposed to a variety of heavy metals (Maitani *et al*. 1996; Leopold 1997), showed that, although Pb exposure induced PC_n, no binding of Pb to PC_n seemed to occur, the Pb being exclusively bound to substances of low molecular weight. Our data show that a large amount of Pb is bound to uncharacterised LMW compounds, nevertheless demonstrate the occurrence of stable Pb-PC_n complexes in *P. tricornutum* exposed to lead.

The literature on Pb-PC_n complexes is very scarce. To our knowledge, this is the first evidence of the separation of native Pb-PC_n complexes. Gupta *et al*. (1995) reported the presence of some thiol-containing Pb-induced peaks, composed by a mixture of PC_2 and PC_3 oligomers, in the SE chromatogram of crude extracts of the aquatic plant *Hydrilla verticillata* exposed to Pb. Based on spectroscopic investigations on *in vitro* reconstructed Pb-PC_3 and Pb-PC_4 complexes, Mehra *et al*. (1995) reported that PC_3 binds one Pb ion per peptide and PC_4 can form two distinct complexes binding either one or two Pb ions per peptide. This finding agrees with our estimation of the metal/peptide ratio of 1.0 ± 0.2 and 1.6 ± 0.1 for Pb-PC_3 and Pb-PC_4 complexes, respectively.

As regards Cd, the occurrence of two Cd-binding complexes, the LMW and HMW form, has been found in Cd-treated plants (Kneer & Zenk 1997; Rauser 2000), macroalgae (Hu & Wu 1998) and yeasts (Winge *et al*. 1992). Further characterization of these complexes has shown that the HMW form was composed by peptides with different chain-length and sulfide (PC-coated Cd-S quantum crystallites). In our experimental conditions, Cd was bound to PC_n oligomers to form distinct families of complexes homogeneous in the chain length of the peptide. We checked the presence of acid-labile sulfide in SEC fractions containing Cd-PC_n complexes by the methylene blue method (Rabinowitz 1978). The concentration of acid-labile sulfide was found to be under the detection limit of the method in each fraction tested, suggesting that, in our conditions of metal exposure, the sulfide incorporation in the metal-phytochelatin complexes doesn't take place. Experiments are in progress to evaluate whether *P. tricornutum* is capable to form PC-coated Cd-S quantum crystallites under metal exposure conditions different from those here reported.

The formation of Cd- and Pb-PC_2 complexes was not demonstrated by our data. This was unexpected, owing to the known binding ability of the PC_2 oligomer. In order to evaluate whether the metal was displaced during the chromatographic separation, *in vitro* reconstructed Cd-PC_2 complexes were injected in the Superdex 30 column. In this case PC_2 eluted associated with Cd, showing that the gel matrix didn't cause any displacement of the metal ion from

the complex. It has been shown that in the metal-phytochelatin complexes the metal binding strength increases with the peptide chainlength (Mehra *et al*. 1995). So, if the metal ion is bound to PC_2 in a weak association, it could be that it was displaced by other cellular compounds capable to bind the metal, during the cellular extract preparation.

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