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Characterization of Hg(II) binding with different length phytochelatins using liquid chromatography and amperometric detection

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ABSTRACT

A simple and rapid methodology is optimised to analyse mixtures of different phytochelatins (PC_n, $n = 2–5$) with Hg(II) by HPLC with amperometric detection as a first step towards the analysis of extracts of plants stressed with Hg(II). The separation was achieved in a C_{18} column with a mobile phase of 0.1% trifluoroacetic acid (TFA) in water and 0.1% TFA in acetonitrile using gradient elution. Electrochemical detection with glassy carbon electrode and UV–vis detection were used in series. This methodology can clearly distinguish between the free peptides and their complexes and permits to study the evolution of the different complexes formed and predicts the possible interactions between the long chain phytochelatin complexes. ESI-MS is used as a complementary technique to find out the stoichiometries of such long chain phytochelatin complexes.

1. Introduction

Mercury is a global pollutant that together with its compounds constitutes one of the most toxic substances found in all environment compartments. It is well known that the incorporation of heavy metals into the cells of plants or animals can induce some mechanisms leading to modulate the possible harmful effects. One of these mechanisms is the synthesis of several compounds such as metallothioneins (MT), in the case of mammalians and some fungi, or phytochelatins (PC), in the case of plants and algae [1–4]. These substances are thiol-rich peptides which interact very efficiently with heavy metal ions minimizing the stress that these could provoke in living organisms. The capability of plants and algae to synthesize this kind of molecules has been used to remove heavy metals from contaminated soils or aquatic systems through a methodology named phytoremediation. The role that PCs play in detoxification mechanism has received great attention in the last decades [1,2,5–7]. These molecules have the general structure of γ-(Glu-Cys)$_n$-Gly where n has been reported as high as 11, but generally is in the range from 2 to 5 [1–4]. They are synthesized enzymatically from glutathione (GSH) by the constitutive enzyme PC synthase.

In the particular case of Hg(II), the high affinity that inorganic forms of this metal ion possess for biomolecules containing sulphydryl groups (–SH) in comparison with other groups like phosphate, carboxyl, amide or amine is well described [8–10]. Due to Hg(II) toxicity, the knowledge of complexation processes with PCs is of great interest. In order to gain deeper insight on the complexation between PCs and heavy metals, different approaches have been developed in the last years. Among them, direct spectrophotometric techniques [11,12] or chromatographic separations combined with spectrophotometric or mass-spectrometric detection techniques have been the most widely used [13–16]. The use of voltammetric techniques or amperometric detection modes has received special attention in the last few years [17–21]. However, little information is still available about the complexation of Hg with PCs or its fragments. Several *in vitro* studies describing the binding of Hg(II) with synthetic PC_n, $n = 2$, 3, 4, or the fragments Cys, Cys-Gly and glutathione (GSH) using differential pulse voltammetry (DPV), electrospray mass-spectrometry (ESI-MS) and isothermal titration calorimetry (ITC) can be stand out [22,23]. In these studies, the interactions of Hg(II) with different thiols are considered independently. In the case of coexistence of different PCs and their fragments, separation procedures are required. Although the separation of the different compounds is not excessively complicated, the way how the detection is done presents more controversy. Thus, Mehra et al. [24] studied the affinity of Hg(II) for GSH, PC_2, PC_3 and PC_4 in synthetic samples using reverse-phase HPLC and UV detection without previous derivatization in conditions in which high sensitivity is not necessary. This detection mode was also used in the study of the stress responses of *Zea mays* to cadmium and mercury [25]. However, no appreciable evidences of PCs were observed in the plants subjected to Hg(II) stress and very poorly defined peaks were obtained in the case of

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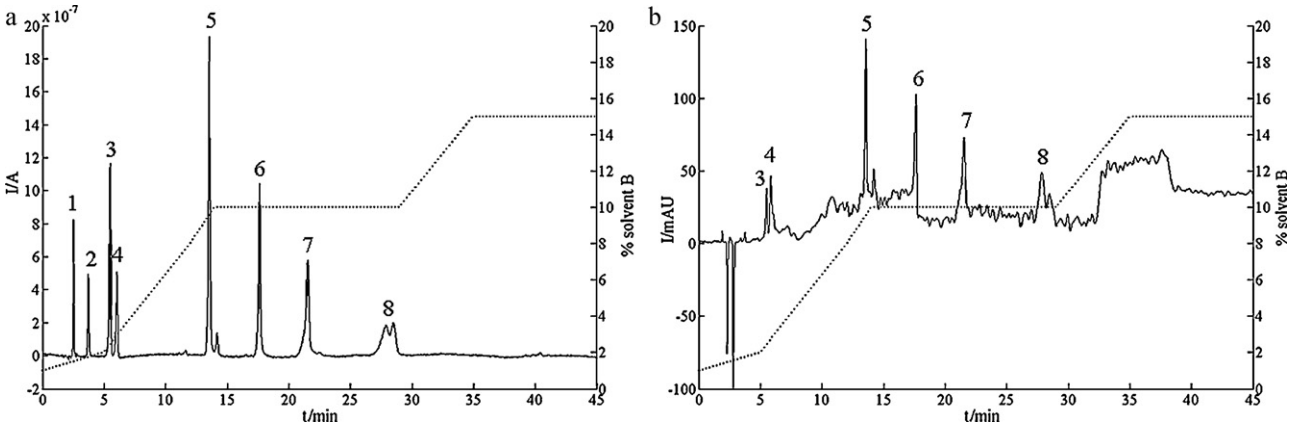

Fig. 1. Chromatograms of Cys (**1**) and Cys-Gly (**2**) at 1×10^{-5} mol L^{-1}, GSH (**3**), γ-Glu-Cys (**4**), PC_2 (**5**), PC_3 (**6**), PC_4 (**7**) and PC_5 (**8**) at 5×10^{-5} mol L^{-1} using a mobile phase of 0.1% TFA/H_2O:0.1% TFA/acetonitrile with gradient profile elution (showed in dotted lines) and a flow rate of 1.2 mL min^{-1} using electrochemical detection (a) at 1.2 V working potential and UV–vis detection (b) at 202 nm wavelength.

Cd(II) presumably due to the high detection limits of this detection mode. Although MS detection is expensive and suffers from difficulties of quantification in certain conditions, it is the most extended method to detect heavy metal-thiol complexes. Krupp et al. [26] applied HPLC separation with simultaneous detection by ICP-MS and ESI-MS to the detection of mercury complexes with Cys and GSH in synthetic samples and in plant extracts spiked with these compounds. Analysis of PCs induced by Hg(II) in natural samples have also been described. Chen and co-workers [27] characterized the Hg-PC complexes in Hg-stressed *Brassica chinensis* L. using reverse-phase HPLC-ESI-MS/MS. This work points out the presence of $HgGS_2$, $HgPC_2$, $HgPC_3$, $HgPC_4$ and Hg_2PC_4 in root extracts of *Brassica chinensis* L. On the same line, the studies by HPLC coupled with ESI-MS/MS and ICP/MS of the extracts of *Brassica napus* grown in the presence of Hg(II) indicate that only PC_2 is found in the plant extracts [28]. As an alternative to UV and MS detection modes, our research group proposed the use of amperometric detection with a glassy carbon electrode [29] as a more appropriate alternative to differentiate between free-thiol compounds and their complexes with mercury. This detection mode takes the advantage of the electroactive character of thiol compounds, the low detection limits that electroanalytical techniques can provide and the economical convenience of this detector in comparison with MS systems. The preliminary application to small thiol peptides [29] yielded satisfactory results and encouraged the application to longer chain peptides such as PCs.

Considering again the Hg-PCs complexes formed in plants stressed with mercury reported elsewhere [25,27,28,30], it is possible to notice that although the studied plants are not the same, the higher PCs are not always observed. This fact, however, is not necessarily a consequence of the non induced formation of these compounds but could be a problem of the formation of some aggregated compounds during the detection step. Nevertheless, it must be taken into account that when synthesis of PCs in plants is activated, PC_2 is firstly formed and the higher PCs (probably PC_3, PC_4 and PC_5) are created later. A tentative study made in our group with an extract of *Hordeum vulgare* roots (results not shown) seems to indicate that a mixture of Hg(II) complexes with PC_n, with n from 2 to 5, could be present, and that signals related to the higher PCs suffer of some uncertainty.

In the present work, a RP-HPLC methodology has been applied to the separation of a mixture of different PCs (from $n = 2$ to $n = 5$), GSH and their fragments. In this study, the formation of different complexes and their separation from the apo-form (not complexed form) has been followed when mercury is added to the solution and in some cases different stoichiometries can be distinguished. ESI-MS has been used to confirm the predicted species. The use of amperometric detection with a glassy carbon electrode allows us to study the evolution of the different complexes formed by each PC depending on the Hg concentration added.

2. Experimental

2.1. Chemicals and reagents

L-Cysteine, γ-Glu-Cys (80% of purity as trifluoroacetate salt), Cys-Gly (85% of purity) and trifluoroacetic acid (TFA) were provided by Sigma–Aldrich (St. Louis, MO, USA). The phytochelatins $(\gamma\text{-Glu-Cys})_n$-Gly ($n = 2$–5), as trifluoroacetate salts, were provided by DiverDrugs S.L. (Barcelona, Spain) with a purity ranging from 86.2% to 99.0%. Glutathione, hydrochloric acid 30%, acetonitrile and methanol were obtained from Merck (Darmstadt, Germany). $HgCl_2$

Table 1
Figures of merit for thiol determination by HPLC-ED (amperometric mode at 1.2 V).

Compound	Retention time (min)	Calibration functions[a]	Detection limit[b]		Quantification limit[c]	
			μmol L^{-1}	μg mL^{-1}	μmol L^{-1}	μg mL^{-1}
Cys	2.47	$A = 0.031c + 8 \times 10^{-9}$, $r^2 = 0.997$	3.21	0.39	10.71	1.30
Cys-Gly	3.47	$A = 0.020c - 6 \times 10^{-8}$, $r^2 = 0.998$	2.90	0.52	9.67	1.72
GSH	4.57	A=0.116$c + 9 \times 10^{-9}$, $r^2 = 0.997$	2.71	0.83	9.02	2.77
γ-Glu-Cys	5.39	$A = 0.147c - 2 \times 10^{-7}$, $r^2 = 0.995$	4.20	1.05	14.00	3.50
PC_2	12.38	$A = 0.193c - 1 \times 10^{-7}$, $r^2 = 0.999$	1.89	1.02	6.32	3.41
PC_3	16.63	$A = 0.108c + 7 \times 10^{-8}$, $r^2 = 0.996$	4.10	3.16	13.66	10.54
PC_4	19.71	$A = 0.202c - 8 \times 10^{-8}$, $r^2 = 0.996$	3.44	3.46	11.47	11.52
PC_5	24.64	$A = 0.230c - 9 \times 10^{-7}$, $r^2 = 0.995$	4.90	6.06	16.33	20.19

[a] A is the peak area (Ampere per second) and c the injected concentration (mol L^{-1}).
[b] Calculated with the 3σ criterion.
[c] Calculated with the 10σ criterion.