Chemometrics applied to the analysis of induced phytochelatins in *Hordeum vulgare* plants stressed with various toxic non-essential metals and metalloids

Àngela Dago, Inmaculada González, Cristina Ariño *, José Manuel Díaz-Cruz, Miquel Esteban

Departament de Química Analítica, Facultat de Química, Universitat de Barcelona (UB), Martí i Franquès, 1-11, E-08028 Barcelona, Spain

ARTICLE INFO

ABSTRACT

Hordeum vulgare plants were stressed with Hg, Cd and As and their phytotoxicity was evaluated in terms of growth inhibition and total metal uptake by the plant. The synthesised phytochelatins ((γ-Glu-Cys)$_n$-Gly, $n = 2$–5; PCs) were determined by HPLC with amperometric detection at a glassy carbon electrode. The results indicate that *H. vulgare* is a good phytostabilisation plant due to its capacity to accumulate heavy metals in roots. Cd and Hg are the most uptake toxic elements, being Cd the most potent inducer of PCs. The data obtained on the different PCs and related peptides induced by each heavy metal were used to perform a Principal Component Analysis (PCA) of the results as a function of the contaminating toxic element or its concentration level. The nature of the stressor element could be predicted from the pattern of PCs and related peptides identified by PCA. PCs were the most strongly induced peptides under Cd and Hg stress, whereas As only tended to synthesise small thiols such as glutathione and γ-glutamylcysteine, both precursors of PCs synthesis. This finding indicates that PCs are induced at different rates depending on the metal stressor used.

1. Introduction

Plants respond to heavy metal stress by inducing SH-containing peptides such as phytochelatins (PCs) [1,2]. Phytochelatins are small, cysteine-rich peptides capable of binding heavy metal ions via thiolate coordination. PCs consist of L-glutamic acid, L-cysteine and a carboxy-terminal glycine. The general structure of these peptides is (γ-Glu-Cys)$_n$-Gly, where n has been reported as being as high as 11, but is generally in the range of 2–5 [3]. Phytochelatins (PCs), which possess a high antioxidant capacity, are thought to be the most important mechanism of detoxification in plants subject to metal stress, and are involved in the accumulation, chelation, sequestration and metabolism of metal ions [4,5]. The PC pathway consists of two parts: metal-activated synthesis of peptides and transport of metal-PC complexes into the vacuole. Glutathione (γ-Glu-Cys-Gly; GSH) is known to be the precursor of phytochelatins, and phytochelatin synthase the enzyme responsible. When plants are exposed to heavy metals in the environment, PCs can reduce the free metal concentration in the cytosol by binding and transporting the metal to specific compartments,

mainly the vacuole, prior to biotransformation into organic compounds or chemical reduction of the element [5].

Several metals have been proved to be inducers of PCs in plants. Cadmium is known to be the most potent PC inducer and is also the most extensively studied metal. A large number of papers have been published on the analysis of PCs in Cd-stressed plants. For example, Bräutigam et al. [6] studied Cd-induced peptides in *Chlamydomonas reinhardtii*, observing several PCs and related peptides by MS detection prior oxidation of the thiols, while Najmanova et al. [7] used RP-HPLC-ED to analyse the abundance of PC species in tissues of *Linum usitatissimum* grown in the presence of Cd(II). They determined PCs content and proved the translocation of Cd–PC complexes from roots to shoots. Mohamed et al. [8] used HPLC with post-column derivatisation to determine PCs in Cd-treated *Brassica juncea*, and demonstrated that GSH and PC biosynthesis increased in both roots and shoots. Many more articles have been published describing the response to Cd stress in plants; however, time-consuming or expensive methodologies were used. Another metal which has been extensively studied is mercury, which is known to be one of the most hazardous pollutants in the environment due to its bioaccumulation and biomagnification in diverse ecosystems. Consequently, decontamination of Hg-polluted sites is a universal goal. Several publications were found which report the content of PCs in Hg-stressed plants.

* Corresponding author. Tel.: +34 93 402 15 45; fax: +34 93 402 12 33.
E-mail address: cristina.arino@ub.edu (C. Ariño).

Chen et al. [9] analysed the generation of PCs in *Brassica chinesis* exposed to different Hg species, and also determined Hg–PC complexes. Iglesia-Turiño et al. [10] analysed PCs in *Brassica napus*, but only found PC_2. In previous studies by our research group [11], *Hordeum vulgare* plants were stressed with Hg or Hg and Cd simultaneously, and PCs from $n=2$ to 5 were detected and quantified. PCs in *Asparagus acutifolius* and *Retama sphaerocarpa* grown in the Almadén mining district, one of the most Hg-contaminated sites in the world, were determined in order to evaluate plant response to Hg pollution (unpublished results).

Arsenic is another contaminant which is widely distributed in the environment. Although it is considered a metalloid rather than a metal, both inorganic species, As(V) and As(III), are highly toxic to plants. For this reason, some authors include arsenic in the term "heavy metal", although strictly speaking it is not a metal [12,13]. As(V) is a phosphate analogue, and it can therefore compete with phosphate in the cytoplasm, replacing it in ATP to form the unstable complex ADP-As, which leads to the disruption of energy flows in cells. Meanwhile, As(III) is highly toxic to plants because it reacts with sulphydryl groups in enzymes and tissue proteins, leading to inhibition of cellular function and death. Although arsenate [As(V)] is the predominant species in aerobic soils, it is thought that once inside the cytoplasm, As(V) is readily reduced to As(III) by GSH, making it the predominant As species in roots and shoots [14,15]. As(III) is then complexed with PCs and subsequently stored in the vacuoles. In a study of PCs induced by As stress, Sneller et al. [16] analysed the short- and long-term toxicity of arsenate in *Silene vulgaris* plants. They reported that PCs accumulation is linearly related to toxicity and can therefore be used as a biomarker for As toxicity; they also proved that PC_2 is the predominant PC in the presence of arsenite, in agreement with Schmöger et al. [17].

Several studies have compared the cellular damage induced in plants according to the metal stressor used. Nevertheless, very few have compared PC content in plant extracts as a function of the metal stressor. Thus, PCs induced by Cd and Hg have been determined by Rellán-Álvarez et al. [18], Ortega-Villasante et al. [19] and Sobrino-Plata et al. [20] in *Zea mays* extracts and in *Medicago sativa*, respectively, demonstrating that Cd is a more potent inducer of PC synthesis than Hg, as inferred from the higher proportions of thiols found at high levels of Cd contamination. However, these authors did not always detect PCs, or if detected they were unable to distinguish between different length PCs and could not determine which PC was the most induced by each metal. In another study comparing PCs induced by Cd and Pb in *Phaeodactylum tricornutum* [21], it was observed that when cells were exposed to Pb, the most abundant PC was PC_2, but PC_4 was the most abundant when Cd was added to the cultures. Nevertheless, the higher capacity of Cd to activate PC synthesis remained unproven.

Other studies have been conducted using different toxic elements; for example, Grill et al. [1] analysed the induction of PCs by Cd(II), Bi (III), As(V), Cu(II), Pb(II), Zn(II) and Ag(I) in *Schizosaccharomyces pombe* cells and showed that Cd is the most potent inducer of PCs. In another multi-metal study conducted by Maitani et al. [22], several metalloids were analysed in addition to metals. Ag(I), As(III), As(V), Cd(II), Cu(II), Ga(III), Hg(II), In(III), Ni(II), Pb(II), Pd(II), Se(IV) and Zn(II) were tested as PC inducers in *Rubia tinctorum* roots. However, this study used a time-consuming HPLC methodology with post-column derivatisation. Moreover, the authors did not describe the PC pattern for each metal or whether there were predominant PCs for each metal or not.

In the present study, barley (*H. vulgare*) was selected as the study plant because it is easy and fast to germinate and is readily available since it is a major cereal grain which is widely cultivated. Hydroponic culture (a method of growing plants using only nutrient solution) is considered a viable research method for phytoremediation studies prior to evaluating the behaviour of plants in greenhouse and/or field soils. Plants were stressed hydroponically with Hg(II), Cd(II) or As(III), and an analysis of the induced PCs was performed using an optimised HPLC methodology with amperometric detection as a sensitive, cheap and fast technique. The results obtained on the different PCs induced by each toxic element were used to apply a more advanced data processing technique in order to identify a pattern in the behaviour observed depending on the contaminating metal and/or its concentration. To this end, chemometrics was applied for the first time in phytoremediation studies. Principal Component Analysis (PCA) was applied to the PC quantification data obtained, with good results. Thus, the aims of this study were (i) to induce PCs in *Hordeum vulgare* plants stressed by different concentrations of several toxic metals and metalloids, (ii) to evaluate heavy metal phytotoxicity in plants, expressed as growth inhibition and total content of these toxic elements in roots and shoots, (iii) to quantify the PCs formed by applying HPLC with amperometric detection using an external calibration curve, and (iv) to apply chemometric tools to the data obtained, in order to evaluate the possible correlation between thiol concentrations in plants and the heavy metal supplied to the plants and/or its concentration level. The results obtained may contribute to improving current knowledge on heavy metal stress response in plants.

2. Material and methods

2.1. Chemicals

L-cysteine, γ-Glu-Cys (80% purity as trifluoroacetate salt) and Cys-Gly (85% purity) were provided by Sigma-Aldrich (St. Louis, MO, USA). Phytochelatins (γ-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 (GSH) and As_2O_6 were obtained from Merck (Darmstadt, Germany). $HgCl_2$ was purchased from Probus (Badalona, Spain). $Cd(NO_3)_2 \cdot 4H_2O$ was obtained from Panreac (Barcelona, Spain).

$Ca(NO_3)_2$, $Fe(NO_3)_3 \cdot 9H_2O$ and $CuSO_4 \cdot 5H_2O$ from Probus, KNO_3, $MnSO_4 \cdot H_2O$ and $ZnCl_2$ from Merck and $Mg(NO_3)_2 \cdot 6H_2O$, KH_2PO_4, H_3BO_3 and $Mo_7O_{24}(NH_4)_6$ from Panreac, were used to prepare the Hoagland solution (nutrient solution).

For plant extract preparation, cleaning the column and preparation of all solutions, ultrapure filtered water obtained from USF Purelab Plus (Hamburg, Germany) was used.

2.2. HPLC instrumentation

An Agilent (Santa Clara, CA, USA) 1100 chromatographic system was used, equipped with a quaternary pump, a Rheodyne 7725i 20 μL loop manual injector (Rohnert Park, CA, USA), a vacuum degasser and a handheld control module. An Ascentis C18 5 μm particle size analytical column measuring 25 cm × 4.6 mm was provided by Supelco (Bellefonte, PA, USA). The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in ultrapure filtered water, pH=2.00, and 0.1% TFA in acetonitrile. Gradient separation was achieved at ambient temperature with a gradient profile as described in Dago et al. [23]. The flow rate was 1.2 mL min^{-1}.

The electrochemical detector (ED) was a CC-5C flow cell BAS (West Lafayette, IN, USA), with a three electrode system and a 0.005 in. gasket, connected to an Autolab PGSTAT 12 (Eco Chemie, Utrecht, the Netherlands). GPES software version 4.9.007 (Eco Chemie) was used for potentiostatic control and data acquisition.

The working electrode was a glassy carbon electrode BAS, the surface of which was polished daily using a suspension of 0.3 μm