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Determination and characterization of cysteine, glutathione and phytochelatins (PC_{2-6}) in *Lolium perenne* L. exposed to Cd stress under ambient and elevated carbon dioxide using HPLC with fluorescence detection

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ABSTRACT

Metal-binding thiols, involved in detoxification mechanisms in plant and other organism under heavy metal stress, are receiving more and more attentions, and various methods have been developed to determine related thiols such as cysteine (Cys), glutathione (GSH) and phytochelatins (PCs). In present study, an HPLC method was established for simultaneous determination of Cys GSH and PC_{2-6} after treatment with disulfide reductant of tris (2-carboxyethyl) phosphine hydrochloride (TCEP) and thiolyte reagent of monobromobimane (mBBr). The separation of thiol derivatives was performed on an Agilent Zorbax Eclipse XDB-C18 column (4.6 mm × 30 mm, 1.8 μm) with a linear gradient elution of 0.1% (v/v) trifluoroacetic acid (TFA)–acetonitrile (ACN) at 0.8 mL min^{-1}. The temperature of the column was maintained at 25 °C. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. The thiol derivatives were well separated in 19 min, and the total analysis time was 30 min. The established method was proved selective, specific and reproducible, and could be applicable to determine Cys, GSH and PC_{2-6} and to evaluate their roles in detoxification mechanisms in Cd-treated *Lolium perenne* L. under ambient and elevated carbon dioxide (CO_2). It was found that the total SH contents and proportions of thiols in roots and shoots were dependent on Cd concentration, whereas the total SH contents decreased and the proportions of thiols altered without significance at elevated CO_2 level.

1. Introduction

Phytochelatins (PCs) are cysteine (Cys)-rich peptides in plants and other organisms with general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2–11) and ability of binding metals through SH groups in the Cys parts [1,2]. Synthesis of PCs in plants under heavy metal stress is considered to be crucial to detoxifying mechanisms [3–6], which has been proved right through inhibitor studies [7–9], biochemical studies [10], mutant analyses [11–14] and gene analyses [15–17]. The synthesis was catalyzed by phytochelatin synthase (PCS) using glutathione (GSH) as substrate [14,15,18–21] and metal ion as activator [1,22,23], and related to species, toxic degree of metal ions, and interactions among metals [5,6,24–29]. It was found that PCs production was direct dependent on aqueous free metal-ions [30] and occurred earlier than any other physiological parameter is affected [5,6,24,25]. Therefore, PCs production could be used as biochemical indicators/markers to assess metal toxicity to biota [6,22,23,31], and the assay of PCs would be a better approach than chemical analyses of metals [5,6,24,31].

Besides being involved in PCs synthesis, Cys and GSH are of importance in plants [3]. Biosynthesis of Cys plays a key role in fixing inorganic sulfur from the environment and provides the only metabolic sulfide donor for the generation of many compounds [32]. GSH is involved in defense against reactive oxygen species (ROS), sequestration of heavy metals, detoxification of xenobiotics, regulation of developmental processes such as cell division and flowering, and furthermore a major transport and storage form of reduced sulfur [33–36]. Consequently, it has become a requirement that methods should be established to determine these thiols and to evaluate their roles in plants and other organisms under heavy metal stress.

A range of methods have been developed for determination of thiols, including electrochemical methods such as cathodic stripping voltammetry and polarography [37–43], capillary electrophoresis (CE) equipped with electrochemical detection [44], laser-induced fluorescence detection [45] or photodiode array

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Table 1
Stock and working standards of thiols (μmol L^{-1}).

Thiols	Stock standard (mmol L^{-1})	Working standards (μmol L^{-1})							
		S1	S2	S3	S4	S5	S6	S7	S8
Cys	100.0000	0.2000	0.5000	1.0000	2.0000	4.0000	8.0000	12.0000	16.0000
GSH	10.0000	0.0500	0.1000	0.5000	2.0000	10.0000	20.0000	50.0000	100.0000
PC_2	1.8498	0.0925	0.1850	0.3700	0.7399	1.4798	2.9597	5.9193	11.8387
PC_3	1.2938	0.0323	0.0647	0.1294	0.6469	1.2938	3.8815	7.7630	12.9383
PC_4	0.9950	0.0249	0.0498	0.0995	0.1990	0.3980	0.7960	1.5920	3.1841
PC_5	0.8081	0.0202	0.0404	0.0808	0.1616	0.3233	0.4849	0.6465	0.8081
PC_6	0.6805	0.0170	0.0340	0.0681	0.1361	0.2722	0.4083	0.5444	0.6805

detection [46,47], and HPLC with UV/vis detection [48,49], electrochemical detection [50–52], fluorescence detections [3,6,28,53–67] or inductively coupled plasma-mass spectrometry (ICP-MS) and electrospray-mass spectrometry (ES-MS) [68–80]. These methods were selective and sensitive for determination of thiols in biological samples and their performance characteristics, advantages and disadvantages were summarized in the previous reviews [53,81–83]. The application of MS, especially tandem MS (MS–MS), could provide more information on thiols in organisms, however, HPLC–MS or MS–MS was not available in most laboratories. Therefore, the method using readily available and low-cost instruments should be established to determine thiols in organisms and to evaluate their roles in detoxifying mechanisms under heavy metal stress.

On the basis of comparing existing methods, an HPLC method with fluorescence detection was established for simultaneous determination of Cys, GSH and PC_{2-6}, and applied to assay their concentrations and to evaluate their roles in *Lolium perenne* L. exposed to Cd stress under ambient and elevated CO_2. Furthermore, since GSH and PC_{2-6} have the same unit of [γ-Glu-Cys], there is a hypothesis that the retention time (*RT*) and response (height) would be dependent on the number (*n*) of SH. The objective of present study therefore was also to estimate the role of *n* on *RT* and height.

2. Experimental

2.1. Chemicals

Trifluoroacetic acid (TFA, ≥99%), γ-cysteine (Cys, ≥99.5%) and glutathione (GSH, ≥97%) were obtained from Fluka (Milwaukee, WI, USA). Monobromobimane (mBBr, ≥95%) was purchased from Fluka (Buchs, Switzerland). Methanesulfonic acid (MSA, 99.5%), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid sodium salt (HEPES, 99.5%) and Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma (Louis, MO, USA). Diethylenetriamine-pentaacetic acid (DTPA, >99%) was purchased from Alfa Aesar (Heysham, Lancs, UK). Phytochelatins (PC_{2-6}, >95%) were obtained from AnaSpec (San Jose, CA, USA). HPLC-grade acetonitrile (ACN) was purchased from Tedia (Fairfield, OH, USA). And all other reagents were analytical-reagent grade. Water was purified by a Milli-Q Gradient system (Millipore Corp., Bedford, MA, USA).

2.2. Preparation of standard and reactant solutions

Extraction buffer containing 0.1% TFA and 5 mmol L^{-1} DTPA was prepared in purified water. Standards of Cys, GSH and PC_{2-6} were used for calibration. Stock standard solutions were separately prepared in extraction buffer, divided into several parts and stored in dark at −20 °C. Fresh working solutions were prepared prior to use with extraction buffer. Appropriate portions of each stock were mixed together and further diluted with extraction buffer to create a series of eight working standards (Table 1). HEPES buffer (200 mmol L^{-1}, pH about 9.0) was prepared in 5 mmol L^{-1} DTPA solution; TCEP solution (20 mmol L^{-1}) was made in HEPES buffer and mBBr solution (50 mmol L^{-1}) was made in ACN. The stock standard solutions was divided into several parts and stored in dark at −80 °C and the other solutions were kept in dark at 4 °C.

2.3. Sample preparation

The sample (approximately 0.20 g), previously stored in dark at −80 °C, was ground in liquid N_2 and the thiols were extracted using 1.8 mL of extraction buffer. After the vigorously mixture, the homogenate was centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatant or standard solutions (250 μL) was transferred and mixed with 650 μL of HEPES buffer and 25 μL of TCEP solution. This reaction mix was pre-incubated at room temperature (25 °C) for 5 min and the derivatization was then carried out by incubating the mix in dark for 30 min at room temperature (25 °C) after the addition of 20 μL of mBBr solution. The reaction was terminated by the addition of 100 μL of 1 mol L^{-1} MSA. The derivatized samples were filtered with 0.20 μm nylon syringe filters (Millipore Corp., Bedford, MA, USA) for HPLC analyses. The whole protocol was quickly carried out in dark.

2.4. Instrumentation

The separation of thiol derivatives was performed using an Agilent Technologies 1200 series HPLC system (Agilent Technologies Inc., Hambruecker Landstrasse, Waghaeusel-Wiesental, Germany) consisting of quaternary pump with degasser, thermostat for ALS/FC/Spotter, thermostatted column compartment, diode array detector, fluorescence detector and autosampler fitted with a 100 μL loop. The column was Agilent Zorbax Eclipse XDB-C18 column (4.6 mm × 30 mm, 1.8 μm; Agilent Technologies Inc., Princeton, MN, USA). The temperature of the column oven was maintained at 25 °C. The excitation and emission wavelengths were set at 380 and 470 nm, respectively. Data were integrated using ChemStation software (Agilent Technologies Inc., Version B.03.02).

2.5. Chromatographic conditions and peak identifications

Derivatized samples (20, 50, or 100 μL) were run with a linear gradient elution. Solvent A was 0.1% (v/v) TFA in water and solvent B was ACN. The flow rate was 0.8 mL min^{-1}. The gradient profile was described as: 0–20 min, 8–26% B; 20–22 min, 26–100% B; 22–24 min, isocratic 100% B; 24–28 min, 100–8% B; 28–30 min, isocratic 8% B, and total analysis time was 30 min. All solvents were filtered with 0.2 μm nylon filter (Nylaflo; Pall Corp., Ann Arbor, MI, USA) and degassed before use.

Identification of peaks from thiol derivatives was performed through comparing the profiles of blank (extraction buffer), individual standards and standards mix, and the thiol concentrations were calculated using the relationship between thiol concentrations in standard solutions and corresponding peak height.