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HPLC with electrochemical detection for studying of synthesis of phytochelatins in cadmium treated flax

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Abstract

If there would be free heavy metals occurring in the organism without control it initiates creation of number of dangerous species (ROS). These species are disturbing of homeostasis of organism by subsequent oxidation of essential cellular structures as nucleic acids. Animal and plant organism has both one efficient defending system which includes synthesis of glutathione (GSH). But plants are limited in case of escape from metal contaminated area. Therefore plant cell has mechanism includes synthesis of phytochelatins (PC) from GSH by phytochelatin synthase. PC´s are able to immobilize heavy metal ion via number of thiol groups. We treated the plants of flax (*Linum usativum*) by various concentration of Cd(II) ions (50, 250, and 500μM) in our study. Subsequently we used our developed method for determination of oxidation stress as GSH/GSSG ratio and levels of PC-2,3,4 and 5. We observed that most synthesised PC under the highest concentration of Cd(II) treatment was PC-2,4 and 5.

Keywords: Heavy metals, phytochelatins, electrochemical detection, reactive oxygen species.

Introduction

The world water and soil contamination is widespread problem which has limited scientific attention because it critically concerns the less developed countries mostly. Nowadays attention is paid to contamination with subsequent link to food chain – extensive pollution of the environment (Gawel et al. 1996; Gawel et al. 2001). The heavy metals are one of the most toxic groups of these undesirable compounds that threaten both plants and animals (Cobbett 2000b; Hall 2002; Zenk 1996).

The management of heavy metal ions inside the plant cell could by partially secured by thiols as glutathione and phytochelatins (Clemens 2001; Cobbett and Goldsbrough 2002; Cobbett 2000a).Phytochelatins ((γ-Glu-Cys)n-Gly) are molecules synthetized by PC-synthase from GSH which structure is (γ-Glu-Cys)-Gly. Name of PC is determined by number of repetitions (γ-Glu-Cys)n in PC structure (n...2,3,4,5 etc.). Then PC2, PC3, PC4 and PC5 could be created. The synthesis of phytochelatins is catalyzed by γ-Glu-Cys dipeptidyl transpeptidase named as phytochelatin synthase (PCS) which is crucial enzyme makes the plant resistant against heavy metal stress (Cobbett 1999; Vatamaniuk et al. 2001). High pressure liquid chromatography with (HPLC) with electrochemical detection (ED) is suitable method for studying of thiol substances due to presence sulfhydryl group SH which is very electro active.

Materials

For purpose of separation and detection of PC 2,3,4 and 5 gradient chromatographic system was employed. System consisted of two chromatographic pumps, autosampler and multichannel coulometric electrochemical detector Coularray. Samples ware prepared according our developed protocol. Single plants of flax which were treated by various concentration of Cd (50, 250, and 500μM) were homogenized by ultrasonic and resolved in 0.2 M phosphate buffer (pH 7.2) and after centrifugation supernatant were injected in chromatographic system.

Method

HPLC-ED system consists of two chromatographic pumps Model 582 ESA (ESA Inc., Chelmsford, MA) (working range 0.001-9.999 ml min^{-1}) and chromatographic column with reverse phase Zorbax eclipse AAA C18 (150 × 4.6; 3,5 μm particles, Agilent Technologies, USA) and twelve-channel CoulArray electrochemical

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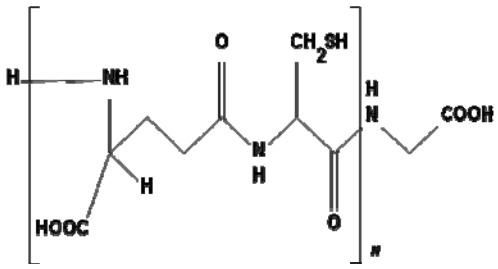

Figure 1: Structure of phytochelatin. Repetition of γ–glutamyl-cysteinyl could be n-2,3,4,5 and everytime is terminated by glycine.

detector (Model 5600A, ESA, USA). Detector consists of three flow analytical chambers (Model 6210, ESA, USA). Each chamber contains four analytical cells. One analytical cell contains two referent (hydrogen-palladium), and two counter and one porous graphite working electrode. Electrochemical detector is situated in control module which is thermostated. Sample (15 µl) was injected by autosampler (Model 542, ESA, USA), which has thermostated space for column. Column was termostated at 30°C. Flow rate of mobile phase was 1 ml min^{-1}. Mobile phase consists of A: trifluoric acid (80 mM) a B: 100% Met-OH. Compounds were eluted by following gradient: 0-7 min (3% B), 7->8 min (15 %B), 8-15 min (15 % B), 15->25 min (30 % B), 25-28 min (98 % B), 28-33 min (98 % B). Detection was carried out at applied potential 900mV.

Results and discussion

We developed fast and rapid method for separation and electrochemical detection of PC 2,4,5 and we were also able to detect amount of GSH and GSSG to. Time of one analysis was 45 minutes including regeneration of the column. Influence of treating by cadmium on generation of ROS is well observed on ratio of GSH and GSSG which is inversed than in normal state.

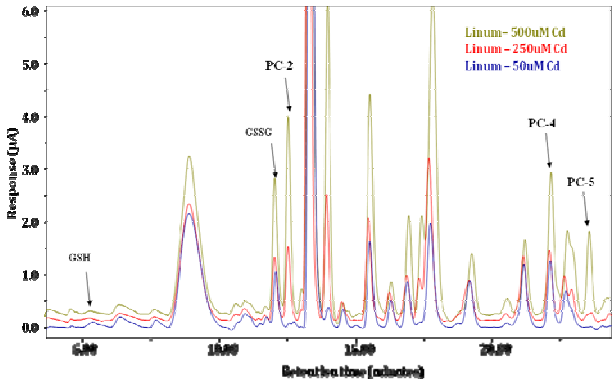

Figure 2: Chromatograms real samples of flax treated by 50, 250 and 500 µM cadmium.

Similarly the PC-2,4 and 5 are increasing with amount of cadmium. Interesting trend was observed in increase of PC-5 which is not commonly preferred form of PC in organism. Forming of PC-5 was increasing markedly due to 500mM cadmium. On the other way we can say that PC-4 could be in the plant even if not treated or if treated less than 50µM cadmium. But occurrence of PC-2 which we hope that is precursor for PC-4 was very sensitive on change of cadmium concentration. Anyway the matrix and biological variability between each plant could not be minimalized and thus we can be assuming but not certainly sure about these trends.

Conclusion

In presented work we optimized the method for determination of six important biological active thiol compounds which are involved in process of metal bonding in plant of flax. We were able to compare amounts of each thiol with applied concentration of cadmium. This method is could be very helpful in biological studies for observation of plant stress on molecular level. Moreower concentration levels of phytochelatins indicate the enzymatic activity of PCS and thus this method could be directly used for selecting of proper plats for bioremediation.

Acknowledgements

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