A NOVEL METHOD FOR ANALYSIS OF PLANTS' RESISTANCE TO HEAVY METALS

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ABSTRACT

Nowadays many less developed countries has many problems concerning about pollution in environment caused by heavy metals as cadmium, lead or mercury. The main aim of this work was to optimize method for determination of activity of main plant protective mechanism against the heavy metals. Sulphur rich peptides phytochlatins (PC´s) are physiologically active compounds because they are able of immobilization of the toxic heavy metal directly in the plant. Most occurring of PC´s is phytochelatin$_2$ (PC-2) which is synthesized by phytochelatin synthase (PCS). We focused on developing of the method based on high performance liquid chromatography coupled with electrochemical detector (HPLC-ED) for determination of PC-2 because it might serve as suitable tool for determination of phytochelatin synthase activity. PCS is the best activated by cadmium ions. We used a model with BY-2 tobacco cells. We conducted the *in vivo* 3 day cultivation experiment where BY-2 cells were treated by various concentrations of cadmium. We then homogenized the cells and immediately analyzed the extracts by optimized HPLC-ED method. Moreover we observed that with higher concentrations of applied cadmium there was increasing of amount of PC-2.

Key words: phytochelatin synthese, coulometric detection, phytochelatin 2, heavy metals

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INTRODUCTION

There is no doubt that heavy metals are occurring in the environment partially due to increasing anthropogenic activities as coal mining or heavy industry is. Especially in less developed countries the landscape is suffering from contamination of growing soil and ground water. Once the heavy metal pollution is demanding of specific area it is wery complicated to dispose it. One of most elegant approach is to employ bioremediation approaches. When the plants with high biomass increment are grown on the demanded place the metal pollution could be very ecologically liquidated. Heavy metals are toxic for both plants and animals [1-3], but plants could avoid the reactive oxygen species generation much effectively than animals. That's because plants owns special biochemical mechanism of creation of phytochelatins. Plant stress peptides as phytochelatins has polymer structure (PC; a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2 to 11)) [4-7]. Phytochelatins can bind heavy metal ions via –SH groups of cysteine units very effectively and consequently transport them to vacuole [5-9], thereby toxicity of the metal is decreased. Biosynthesis of Phytochelatins is catalyzed by γ-Glu-Cys dipeptidyl transpeptidase (EC 2.3.2.15), which has been named as phytochelatin synthase (PCS) [10-11]. The mechanism of the creation of polymer structure of higher PC´s than PC_2 applied in case of biosynthesis of PC-3,4 or 5 but every time a GSH is donor and PC is an acceptor of γ-Glu-Cys dipeptide (details see in Fig. 1).

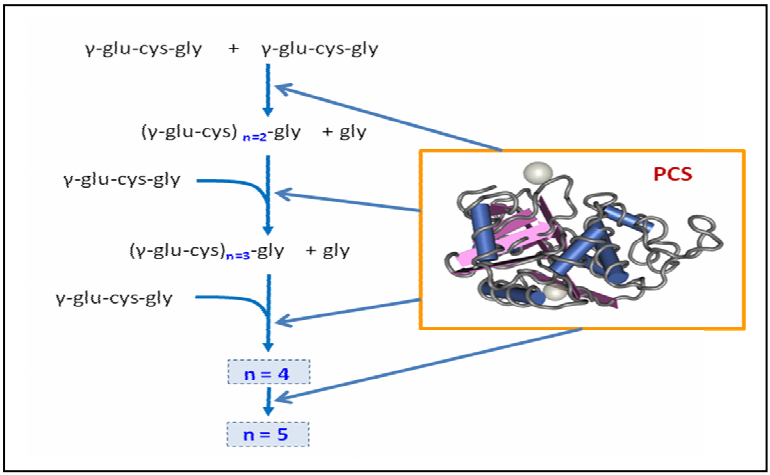

Fig. 1 Scheme of Fytochelatin synthase functions.

We attempted to employ an HPLC method with coulometric detection for analysis of GSH and PC_2 simultaneously in Cell BY-2 Tobacco extract. In connection with HPLC the coulometric detector is one of the most suitable because of its sensitivity, low noise background and possibility in baseline correction application which is needed if gradient elution is applied. Moreover the higher area of the working electrode which is made from porous graphite is capable to oxidise or reduce more than 90% of the analyzed substance. And this is more than classic graphite planar electrodes in flow arrangement [18]. Moreover electrochemical techniques generally as differential pulse and cyclic voltammetry are suitable and sensitive methods for detection of thiols [12-17].

EXPERIMENTAL

Working standard solutions were prepared daily by dilution of the stock solutions. All solutions were filtered through 0.45 µm Nylon filter discs (Millipore, Billerica, Mass., USA) prior to HPLC analysis. Stock standard solutions of the thiols (1 $mg.ml^{-1}$) were prepared with ACS water (Sigma-Aldrich, USA) and stored in dark at -20 °C. Reduced (GSH) and oxidized (GSSG) glutathione, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, USA). Phytochelatin$_2$ (PC_2) $(\gamma\text{-Glu-Cys})_2$-Gly was synthesized in Clonestar Biotech (Brno, Czech Republic) with a purity above 90 %. HPLC-grade methanol (>99.9%; *v/v*) was from Merck (Dortmund, Germany) were used. Other chemicals were purchased from Sigma-Aldrich (St. Louis, USA) unless noted otherwise. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany), controlled by software MultiLab Pilot; Weilheim, Germany. The pH-electrode (SenTix H, pH 0..14/0..100°C/3mol.l^{-1} KCl) was regularly calibrated by set of WTW buffers (Weilheim, Germany). HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001-9.999 $ml.min^{-1}$ (Model 582 ESA Inc., Chelmsford, MA), Zorbax eclipse AAA C18 (150 × 4.6; 3,5 µm particles, Agilent Technologies, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The sample (20 µl) was injected using autosampler (Model 542, ESA, USA).

METHOD DEVELOPMENT

Firstly we tested the electrochemical detector response for PC-2 and influence of different concentration of methanol in mobile phase (MF). Wefound that the oxidation maximum provided from constructed hydrodynamic voltammogram is changing due to change of methanol amount in (MF). Obtained data were considered during the optimisation of the separation method. We optimized the chromatographic method for separation of glutathiones reduced (GSH), as substrate of reaction, oxidized glutathione (GSSG) as control of stability of GSH in the substrate and PC-2. We reached very good resolution in separation of all compound of interest. The PC-2 as key compound has a retention time about 10.7 minutes and its peak wasn't coeluted with other compounds neither in the real sample of cell extract. During the main *In vivo* experiment the BY-2 Tobacco cells in liquid medium were treated by $Cd(NO_3)_2$ in various increasing concentrations 0, 5,

10, 25, 50 and 100 µM. After 3 days of cultivation Cells were harvested and in same time centrifuged 5 minutes (360g) to remove the liquid medium and the cells were immediately homogenized in mortar by liquid nitrogen. After 2 minutes of homogenisation the phosphate buffer with 1mM TCEP was added and whole mixture was homogenized another 2 minutes. After 20 minutes of centrifugation (10,000g) we obtained supernatant which was initial solution for further tests of activity of PCS. The supernatant was divided to seven aliquots of 100 µl of the cell extract. Immediately we added a various concentrations of reduced glutathione (GSH) (0, 0.05, 0.1, 0.25, 0.5, 1.0, 2.5 and 5.0 mM) as a substrate for the PCS reaction. All liquid handling operations were conducted on the ice. Than cadmium(II) ions (50 µM $Cd(NO_3)_2$) were added for initializing of PCS activity. We optimized that mixtures should be incubated at 35 °C for 30 min for obtaining the highest yield of PC-2. Using the optimized separation method on HPLC-ED, PC_2 was determined. The signal of PC-2 was increasing with increase of applied concentration of GSH. The highest activity of PCS as 278 fkat was determined in cells treated with 100 Cd(II) ions. The simplified scheme of the sample preparation is shown (Fig. 2).

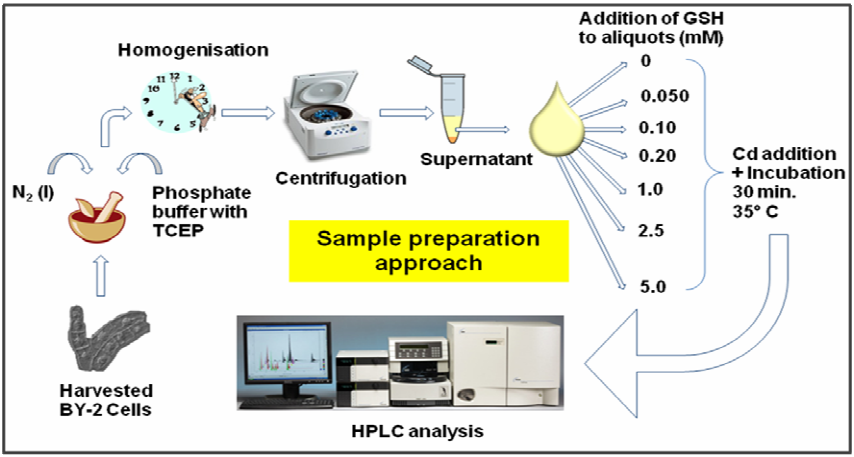

Fig. 2 Scheme of preparation of the sample.

CONCLUSIONS

The HPLC-ED method for detection of PC-2 has been optimized. We used the multichannel coulometric detection which enabled us to ease the sensitivity of detection to 340 femtomoles per injection of PC-2. Than we designed and partially adopted the sample preparation approach for analysis of the series of cells extracts treated by different concentrations of cadmium. From different found concentrations of PC-2 regards to GSH scale concentration added we were able to precisily determine the PCS activity for differen samples. The cells treated with 100 µM Cd(II) ions had more than seven times active PCS compared to control ones. These results are in well agreement with those published by Nakazawa et al. [19] and Ogawa et al. [20]. We proved that our

method can be useful for determination of PCS activity when the plant is treated by various concentration of the metal. Thus this approach can serve as standard method for determination of plant durability against heavy metals in polluted environment.

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