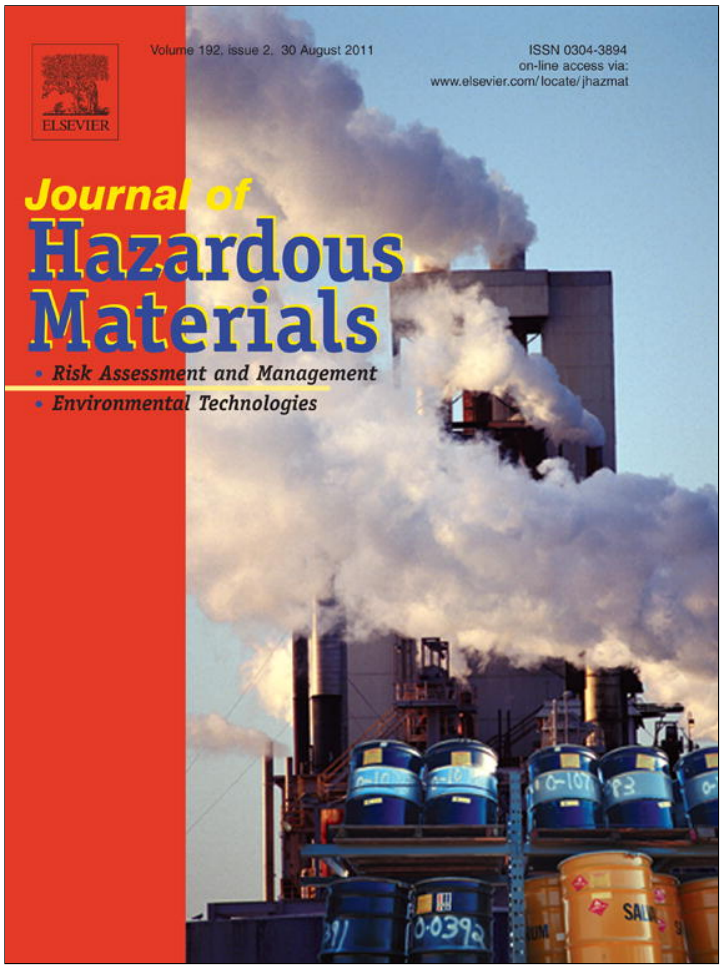

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Phytochelatin synthase activity as a marker of metal pollution

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ABSTRACT

The synthesis of phytochelatins is catalyzed by γ-Glu-Cys dipeptidyl transpeptidase called phytochelatin synthase (PCS). Aim of this study was to suggest a new tool for determination of phytochelatin synthase activity in the tobacco BY-2 cells treated with different concentrations of the Cd(II). After the optimization steps, an experiment on BY-2 cells exposed to different concentrations of $Cd(NO_3)_2$ for 3 days was performed. At the end of the experiment, cells were harvested and homogenized. Reduced glutathione and cadmium (II) ions were added to the cell suspension supernatant. These mixtures were incubated at 35 °C for 30 min and analysed using high performance liquid chromatography coupled with electrochemical detector (HPLC-ED). The results revealed that PCS activity rises markedly with increasing concentration of cadmium (II) ions. The lowest concentration of the toxic metal ions caused almost three fold increase in PCS activity as compared to control samples. The activity of PCS (270 fkat) in treated cells was more than seven times higher in comparison to control ones. K_m for PCS was estimated as 2.3 mM.

1. Introduction

United Nations Environment Programme (UNEP) is a primary driving force in the UN system for international activities related to the sound management of chemicals. One of the last UNEP councils was devoted to lead and cadmium as representatives of dangerous pollutants (Fig. 1). The intensive use of wastewater irrigation, sewage sludge, pesticide and emissions from vehicle exhausts, mining, smelting and the rapid development of industries without effective control has resulted in a large accumulation of these heavy metal ions in soils [1–3]. Heavy metals, unlike organic contaminants, are not degradable and persistent in soils [4]. Due to possibility of accumulation of these toxic metal ions in food chain, an action to remediate polluted soils is very needed. Conventional methods for the removal of the heavy metals ions from environment include mainly soil excavation and disposal to landfill, chemical precipitation, electro flotation, ion exchange and reverse osmosis, adsorption onto activated carbon [5]. However, there have been suggesting and testing new methods for remediation of polluted environment based on the organisms, which include bioremediation [6] and/or phytoremediation [7–11]. Phytoremediation employs plants to cleanse the nature, as plants can absorb, accumulate and detoxify contaminants of their substrates (soil, water and air) through physical, chemical or biological processes [11]. Various soil and plant factors such as the physical and chemical properties of the soil, the plant and microbial exudates, bioavailability of metals, and the ability of plants to uptake, accumulate, translocate, sequester and detoxify metals account for phytoremediation efficiency [12]. Understanding of the mechanisms of plant tolerance to a particular metal is important for developing plants that are suitable for phytoremediation of the contaminated sites [13]. Moreover, enhancing of some mechanisms of plant tolerance to metal-polluted environment using genetic engineering may be of great potential [14].

Phytochelatins (PC; a basic formula $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ ($n = 2–11$)) are one of the most studied plant stress peptides participating in the detoxification of heavy metals (Fig. 1). They are able to bind heavy metal ions via –SH groups of cysteine units and consequently transport them to vacuole [15,16], where an immediate toxicity do not menace yet. The synthesis of phytochelatins pro-

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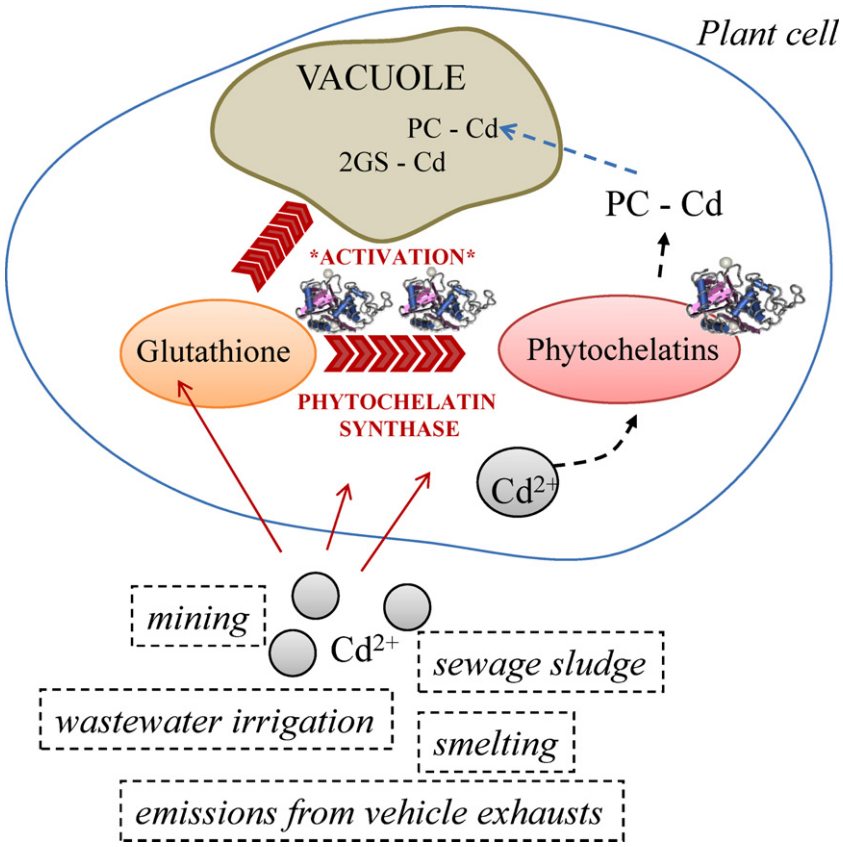

Fig. 1. The intensive use of wastewater irrigation, sewage sludge, pesticide and emissions from vehicle exhausts, mining, smelting and the rapid development of industries without effective control has resulted in a large accumulation of these heavy metal ions including cadmium (II) in soils. Cadmium (II) ions enter a plant cell, where these ions trigger protective mechanisms including synthesis of phytochelatins. Glutathione serves as a precursor of these peptides, which are composed of two or more repeating γ-Glu-Cys units with a terminal glycine residue; $(\gamma\text{-Glu-Cys})_n$-Gly, where $n = 2–11$. The enzyme responsible for the synthesis of these peptides is known as phytochelatin synthase (glutathione γ-Glu-Cys transferase or γ-Glu-Cys dipeptidyl transpeptidase), which is a constitutive enzyme that is activated by cadmium and other metal ions. The structure of the enzyme was done according to Vivares et al. [46].

ceeds from glutathione by transferring γ-Glu-Cys moiety from a donor to an acceptor molecule catalyzed by γ-Glu-Cys dipeptidyl transpeptidase (EC 2.3.2.15) called phytochelatin synthase (PCS). PCS activity in the plant cell exposed to heavy metal can be studied by determination of increasing phytochelatins concentration.

Low molecular compounds rich in thiols including PCs can be determined by numerous methods [17–21], however, each method has its advantages and limitations and may serve a particular need in analysis. UV detection suffers from difficulties associated with insufficient selectivity, whereas using mass detection it may be difficult to quantify all thiol compounds in a single chromatogram. In contrast, electrochemical detection (ED) is an attractive alternative method for electroactive species detection, because of its inherent advantages of simplicity, ease of miniaturization, high sensitivity and relatively low cost. If electrochemical detector is coupled with effective separation method such as high performance liquid chromatography (HPLC) [22–30], capillary electrophoresis [31,32] and/or capillary liquid chromatography [33], real sample can analysed with high sensitivity and selectivity.

The aim of this study was to suggest a new tool (high performance liquid chromatography coupled with electrochemical detector) for determination of the phytochelatin synthase activity. The optimized procedure was subsequently used for studying PCS activity in the tobacco BY-2 cells treated with different concentrations of cadmium (II) ions.

2. Experimental

2.1. Chemicals and pH measurements

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). Other chemicals were purchased from Sigma–Aldrich (St. Louis, USA) unless noted otherwise. Stock standard solutions of the thiols ($1\,mg\,ml^{-1}$) were prepared with ACS water (Sigma–Aldrich, USA) and stored in dark at $-20\,^{\circ}C$. 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. The pH value was measured using WTW inoLab Level 3 with terminal Level 3 (Weilheim, Germany).

2.2. High performance liquid chromatography with electrochemical detection

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 electrochemical detector

includes one flow cell (Model 6210, ESA, USA). The cell consists of four analytical cells containing working carbon porous electrode, two auxiliary and two reference electrodes. The sample (20 μl) was injected using autosampler (Model 540 Microtiter HPLC, ESA, USA).

2.3. Plant cell culture

The suspension culture of tobacco *Nicotiana tabacum* cv. BY-2 was grown in liquid Murashige and Skoog medium (MS medium Micro and Macro elements, Duchefa, The Netherlands) supplemented with sucrose (30 g l^{-1}), KH_2PO_4 (0.2 g l^{-1}), thiamine (1 mg l^{-1}) and 2,4-dichlorophenoxyaetic acid (0.2 mg l^{-1}) (all from Duchefa, Plant cell tested, The Netherlands) according to Nagata et al. [34]. The suspension cultures (20 ml) were grown in 50 ml Erlenmeyer flasks at 27 °C with shaking at 135 rpm (ES-20, Biosan, Latvia). Subcultivation of culture was performed after 3 or 4 days by transferring 2 or 1 ml, respectively, of suspension culture into a fresh medium (total volume of 20 ml). One day old suspension cultures were treated with 0, 5, 10, 25, 50, and 100 μM $Cd(NO_3)_2$ and were cultivated for 3 days in the medium at 27 °C with shaking at 135 rpm.

2.4. Esterase assay

Cultivation medium of tobacco BY-2 cells was removed by centrifugation (360 *g*; 5 min; 20 °C; centrifuge MR 22, Jouan, USA). The cells were washed twice in 50 mM potassium phosphate buffer (pH 8.7). The washed BY-2 cells and harvested ESEs (100–200 mg, respectively) were mixed with extraction buffer (250 mM potassium phosphate, pH 8.7) to a final volume of 1 ml and homogenized using a Potter-Elvehjem homogenizator (Kavalier, Czech Republic) placed in an ice bath for 10 min. The redox state of the obtained solution was maintained by addition of 1 mM dithiothreitol (DTT). The homogenized samples were sonicated for 1 min in an ice bath using a Transsonic T310 sonicator (Czech Republic). The homogenate was centrifuged at 10,000 *g*; 15 min; 4 °C (centrifuge MR 22, Jouan, USA). An aliquot (5–20 μl) of the supernatant was mixed with potassium phosphate buffer (995–980 μl, 1 M, pH 8.75). The reaction was started by the addition of FDA to final concentration of 5 μM. The final volume of the reaction mixture was 1 ml. As a blank sample an equal volume of extraction buffer was used.

After incubation (15 min, 45 °C, dry block, Major Science, Taiwan), an aliquot (5–20 μl) of the reaction mixture was added to 5 mM potassium phosphate buffer (pH 8.7, 1980–1995 μl). The fluorescence ($\lambda_{excitation}$ 490 nm and $\lambda_{emission}$ 514 nm) was read immediately using a spectrofluorimetric detector RF-551 (Shimadzu, USA). A stock solution of FDA was prepared in acetone dried by anhydrous calcium chloride. The amount of acetone did not exceed 1% (v/v) in the reaction mixture. Esterase activity in international units (IU, one unit liberates 1 μmol of fluorescein per minute under specified conditions) was recalculated to relative units (100% represents the highest activity measured in an experiment) [35,36].

2.5. Detection of cadmium

Differential pulse voltammetric measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland), using a standard cell with three electrodes. The three-electrode system consisted of hanging mercury drop electrode (HMDE) as working electrode, an Ag/AgCl/3 M KCl reference electrode and a glassy carbon auxiliary electrode. For smoothing and baseline correction the software GPES 4.9 supplied by EcoChemie was employed. Acetate buffer (0.2 M CH_3COOH + 0.2 M CH_3COONa) was used as the supporting electrolyte. The measurements were performed at room temperature. The samples were deoxygenated prior to the measurements by purging with argon (99.999%) saturated with water for 10 min. Cadmium concentration was determined by a differential pulse adsorptive stripping voltammetric method. The anodic scan was initialized at −0.7 V and stopped at the −0.4 V. The cadmium was deposited on HMDE at potential −0.7 V with accumulation time of 180 s at room temperature. The solution was stirred (1450 rpm) during deposition process. Other parameters of method were: modulation time 0.02 s, interval time 0.1 s, step potential 1.05 mV scan rate 10.5 mV s^{-1}, modulation amplitude 49.5 mV [37,38].

To prepare the samples, microwave digestion were used according to Majzlik et al. [39]. Briefly, the mineralization of samples took place in a microwave system Multiwave3000 (Anton-Paar GmbH, Austria). Cells and/or cultivation medium without cells (100 μl) were placed into glass vials MG5 and 900 μl of nitric acid (w/w, 65%) was added. Prepared samples were sealed and placed into the rotor 64MG5 (Anton-Paar GmbH, Austria). Rotor with the samples was inserted into the microwave system and the microwave digestion was carried out under the following conditions: power 100 W, ramp 10 min, hold 99 min, cooling 10 min, maximum temperature 80 °C. Sample preparation for subsequent electrochemical measurements was as follows: 15 μl mineralized sample was pipetted into Eppendorf tubes with 985 μl acetate buffer (pH 5.00). A blank digestion was simultaneously carried out in the same way.

2.6. Determination of phytochelatin synthase activity

At the end of 3-day long cultivation, approx. 500 μg of BY-2 cells were harvested in 10 ml of the cultivation medium in all experimental groups. The media containing cells were centrifuged at 2000 rpm and 4 °C. The cells were further washed with 10 ml of 20 mM phosphate buffer (pH 7.5) for 10 min and centrifuged for 15 min (2000 rpm, 4 °C). Supernatant was removed and 200 μl of cells were transferred to a test-tube and liquid nitrogen was added. The samples were frozen to disrupt the cells. Then, 800 μl of 0.2 M phosphate buffer (pH 7.2) was added into the test tube. The mixture was processed by hand-operated homogenizer ULTRA-TURRAX T8 (IKA, Germany) at 25,000 rpm for 3 min [40]. The homogenate was then transferred to a new test-tube. The mixture was further homogenized by shaking on a Vortex-2 Genie (Scientific Industries, New York, USA) at 4 °C for 30 min. The homogenate was centrifuged (16,400 *g*) for 15 min at 4 °C using a Universal 32 R centrifuge (Hettich-Zentrifugen GmbH, Tuttlingen, Germany). Prior to analysis the supernatant was filtered through a membrane filter (0.45 μm Nylon filter disk, Millipore, Billerica, Mass., USA) and used for determination of PC_2 and activity of phytochelatin synthase.

The protocol for determination of phytochelatin synthase activity was adopted from Nakazawa et al. [41] and modified. Briefly, reduced glutathione as a substrate of the enzyme reaction (various concentration) and cadmium (II) ions (50 μM $Cd(NO_3)_2$) as an activator of PCS were added to the cell suspension supernatant (100 μl). These mixtures were incubated at 35 °C for various times (Eppendorf 5402, USA). The addition of 4 μl 5-sulfosalycilic acid (30%, w/w) stopped the enzyme reaction. The supernatant was filtered through a membrane filter (0.45 μm, Millipore) before injection into the reversed-phase HPLC column. The amount of synthesized PC_2 correlated with PCS activity. Other experimental parameters were optimized as follows: both the detector and the column were kept at 30 °C. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100% methanol. Compounds were eluted by the following linear increasing gradient: 0–1 min (3% of B), 1–12 min (20% of B), 12–15 min (98% of B), and 15–20 min (98% of B). Flow rate of the mobile phase was 1 ml min^{-1}. Detection was carried out at applied potential 900 mV applied on four electrodes.

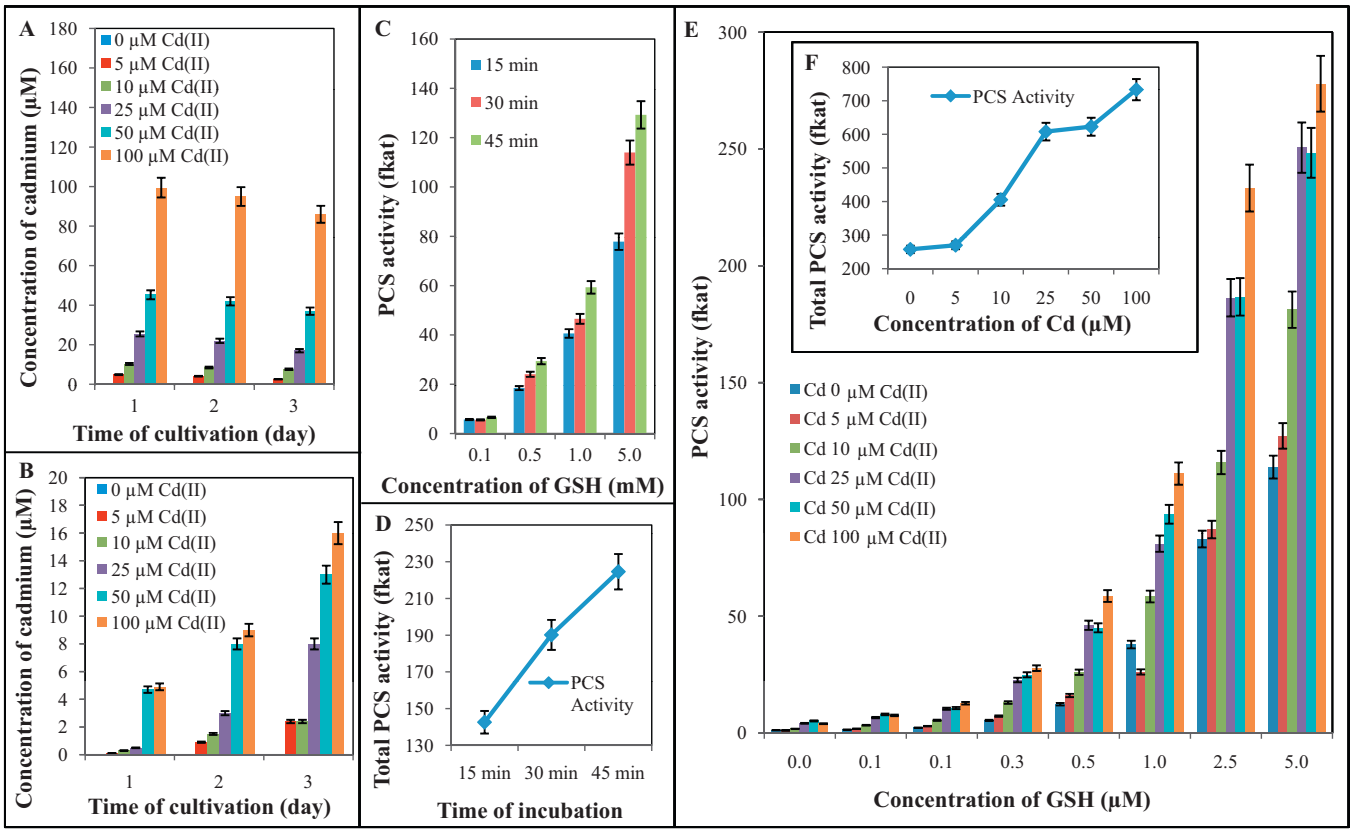

Fig. 2. Content of cadmium (II) ions in (A) cultivation media and in (B) tobacco BY-2 cells treated with cadmium (II) ions for 3 day. Content of cadmium (II) ions in both matrices was determined by differential pulse adsorptive stripping voltammetry. (C) Dependence of concentration of PC_2 on GSH addition. Content of PC_2 linearly enhances with addition of GSH and increasing incubation time. The highest response gives addition 5 mM GSH and incubation for 45 min. (D) Dependence of sums of PCS activities on time of the cultivation. (E) Chromatograms of cell extracts treated with various concentration of cadmium (II) ions. Concentration of PC_2 and thus PCS activity enhance with increasing concentration of cadmium (II) ions. PCS achieves the highest activity in cells treated with 100 μM cadmium (II). (F) Dependence of sums of PCS activities on concentration of cadmium (II) ions. Experimental parameters. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100% methanol. Linearly increasing gradient: 0–1 min (3% of B), 1–12 min (20% of B), 12–15 min (98% of B), 15–20 min (98% of B). Flow rate 1 ml min^{-1}. Potential 900 mV.

3. Results and discussion

Tobacco BY-2 cells were exposed to different concentrations of 0, 5, 10, 25, 50 and 100 μM $Cd(NO_3)_2$. These cells were cultivated according to conditions shown in Section 2 for 3 days. Primarily, the growth of the treated cells was estimated using esterase assay. Activity of these enzymes was determined using flourimetrically using esterase assay as described in Section 2. The activities of esterase were determined in the first, second and third day of the treatment. It was found previously that activity of these enzymes connects with the growth of cell suspension [35,36,42–44]. Therefore, dependencies of esterase activity on day of the treatment were plotted with linear regression with the resulting linear regressions as follows: 0 μM Cd(II) – $y = 40x - 21$; 5 μM Cd(II) – $y = 33.5x - 17.33$; 10 μM Cd(II) – $y = 31x - 18$; 25 μM Cd(II) – $y = 29.5x - 19$; 50 μM Cd(II) – $y = 23.5x - 17.66$; and 100 μM Cd(II) – $y = 18.5x - 15$. It clearly follows from the results obtained, mainly based on slopes of the straight lines that cadmium (II) ions had adverse effect on the growth of the cells, which enhanced with the increasing time of the treatment and applied concentration of toxic ions. The growth of the cells treated with the highest concentration of cadmium (II) ions were depressed for more than 50% compared to control cells.

Further, differential pulse adsorptive stripping voltammetry was employed for determination of cadmium (II) ions accumulated by tobacco cells during the treatment (Fig. 2A). Concentration of accumulated cadmium (II) ions enhanced with the increasing time of the treatment and applied concentration, which can be closely related with poor growth of the cells. In addition, concentration of cadmium (II) ions in the cultivation media, in which the cells were incubated, was determined (Fig. 2B). The concentration of cadmium (II) ions in medium had the opposite trend compared with the concentration of these toxic ions in tobacoo BY-2 cells. The concentration of cadmium (II) ions decreased with the increasing time of treatment in media. This phenomenon closely connects with the increasing concentration of cadmium (II) ions in cells.

3.1. Optimization of method for determination of PCS

Spectrometric protocols are often used for determination of enzyme activities in various types of samples. In certain specific cases, such protocols are costly due to high demands on fluorescently labelled substrates or laborious sample purification. Nakazawa et al. showed simple and easy-to-use method for determination of PCS activity in plant cell extract. It is based on detection of PC_2 content before and after activation by cadmium (II) ions in the presence of reduced glutathione as a substrate for PCS [41]. Similar assay was also published by Ogawa et al. [45], who used different labels for determination of PC_2. Both scientific research groups attempted to express PCS activities. Nakazawa et al. evaluated differences between various experimental variants in %; Ogawa et al. expressed PCS activity as μmol min^{-1} mg^{-1} protein. In this study, we adopted the second protocol and combined HPLC-ED technique for determination of PC_2, which has not been done before. The content of PC_2 was re-calculated on PCS expressed in SI units, katals, which approach also has not been used before. More-

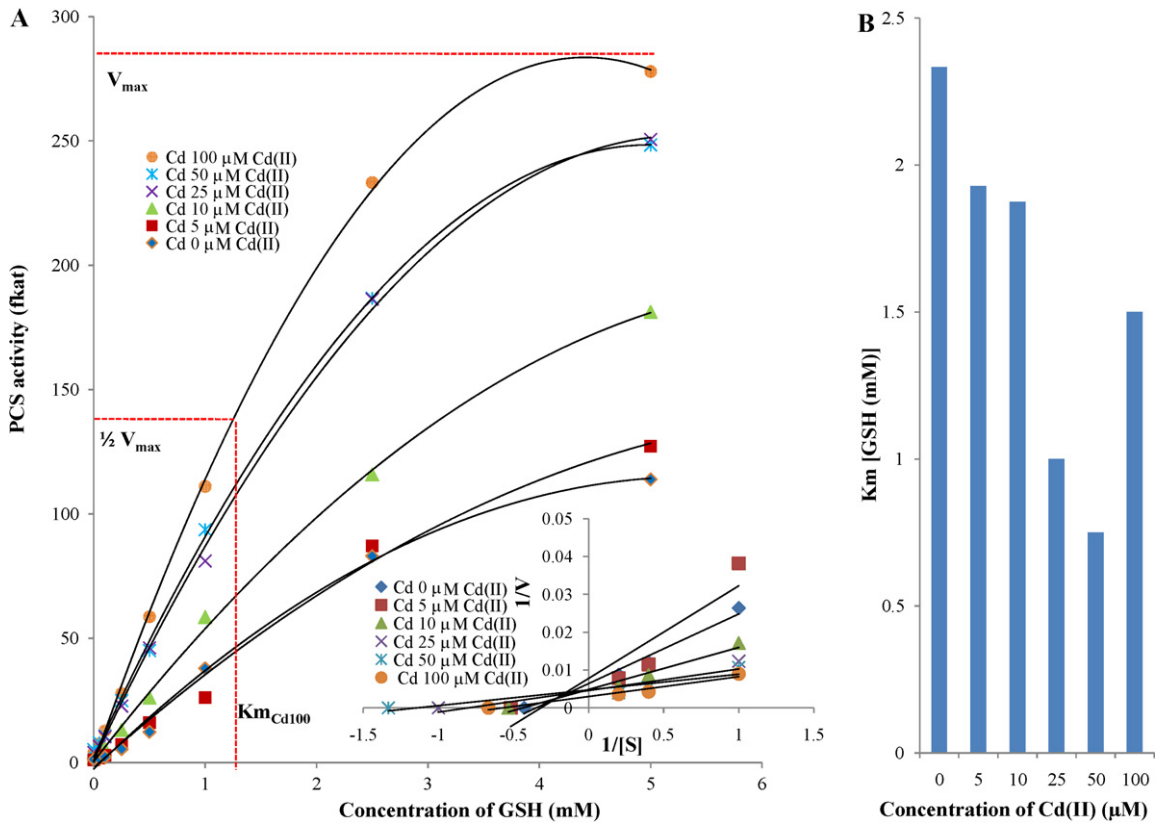

Fig. 3. (A) K_m of PCS for GSH determined for all applied concentrations of cadmium (II) ions in tobacco cells extracts by saturation curve and by (inset A) Lineweaver–Burk plot. (B) Calculated K_m. The Michaelis constant is equivalent to the substrate concentration at which the rate of conversion is half of V_{max} (maximum rate of an enzyme reaction). K_m approximates the affinity of enzyme for the substrate. A small K_m indicates high affinity, and a substrate with a smaller K_m will approach V_{max} more quickly.

over, experimental parameters as concentration of substrate, time of incubation and temperature of sample storage were optimized.

Because reduced glutathione is a substrate for PCS and based on the abovementioned optimized conditions, the following chromatographic parameters were selected. As previously published [22,24,25], these parameters were used for detection of GSH, GSSG and PC_2 simultaneously with the highest sensitivity for PC_2. Both the detector and the column were thermostatically controlled at 30 °C. Mobile phase consisted of A: trifluoroacetic acid (80 mM) and B: 100% methanol. Compounds were eluted by the following linearly increasing gradient: 0–1 min (3% of B), 1–12 min (20% of B), 12–15 min (98% of B), and 15–20 min (98% of B). Flow rate of the mobile phase was 1 ml min^{-1}. Detection was carried out at potential 900 mV applied to four working electrodes. Duration of one analysis was 15 min, which is shorter than in other published methods [22,24].

3.2. Concentration of substrate and time of incubation

Reduced glutathione as a substrate of the enzyme reaction (0.1, 0.5, 1 and 5 mM) and cadmium (II) ions (50 µM $Cd(NO_3)_2$) as an activator of PCS were added to the cell suspension supernatant (100 µl). These mixtures were incubated at 35 °C for 15, 30 and/or 45 min to optimize concentration of the substrate and incubation time simultaneously. The addition of 4 µl of 5-sulfosalycilic acid (1 M) stopped the enzyme reaction. After the stopping the reaction, the content of PC_2 was immediately determined in the obtained samples using HPLC-ED. The results are shown in Fig. 2C. It is clearly visible that the content of PC_2 enhanced with increasing concentration of the substrate, which correlates well with the fact that PCS occurred in real samples in active form. To optimize the incubation time, all measured dependencies were plotted with linear regression as 15 min: PCS activity = $23.85c_{GSH} - 23.99$, 30 min: PCS activity = $34.76c_{GSH} - 39.38$ and 45 min: PCS activity = $39.79c_{GSH} - 43.34$. The sums of PCS activities depending on time of the cultivation are shown in Fig. 2D. The higher the slope of regression curve is, the higher activity of PCS is. Incubation time of 30 min was selected for the following experiments since longer incubation time did not cause marked change in the content of PC_2 (app. 15%) as compared to shorter incubation (increase for more than 30%).

3.3. Activity of PCS in BY-2 cells treated with cadmium (II) ions

The activity of PCS was determined in BY-2 cells exposed to different concentrations of 0, 5, 10, 25, 50 and 100 µM $Cd(NO_3)_2$. Defined amount of the cells were harvested from all experimental groups at the end of the treatment (on the third day of the treatment) and homogenized according to protocol overviewed in Section 2.6. Reduced glutathione (0, 0.05, 0.1, 0.25, 0.5, 1, 2 and 5 mM) and cadmium (II) ions (50 µM $Cd(NO_3)_2$) were added to the cell suspension supernatant (100 µl). The concentration interval of added GSH was extended in order to describe better the effect of cadmium (II) ions treatment on BY-2 tobacco cells. These mixtures were incubated at 35 °C for 30 min. The PC_2 signal enhancing with increasing concentration of GSH was detected. Based on these analyses, dependencies of PC_2 concentration on GSH concentration were determined and are shown in Fig. 2E. The obtained linear regressions can be described by the following equations for the individual applied concentrations of cadmium (II) ions: (i) 0 µM PCS activity = $7.566c_{GSH} - 18.42$; (ii) 5 µM PCS activity = $8.012c_{GSH} - 19.68$; (iii) 10 µM PCS activity = $11.49c_{GSH} - 27.13$; (iv) 25 µM PCS activity = $16.51c_{GSH} - 37.43$; (v) 50 µM PCS activity = $16.54c_{GSH} - 36.68$; and (vi) 100 µM PCS

activity = $19.47c_{GSH} - 43.16$. The concentration of PC_2 and thus PCS activity increased markedly with increasing concentration of cadmium (II) ions as well as the slopes of the equations. The lowest concentration of the toxic metal ions caused almost three times enhancement of PCS activity as compared to control samples. The highest activity of PCS (278 fkat) was determined in cells treated with 100 Cd(II) ions. This clearly indicates dependence of sums of PCS activities on concentration of cadmium (II) ions (Fig. 2F). The cells treated with the highest concentration had more than seven times more active PCS compared to control cells. These results are in good agreement with those published by Nakazawa et al. [41] and Ogawa et al. [45]. In addition, it can be concluded that the enhancing PCS activity with increasing concentration of toxic metal ions and time of the treatment relates to the fact that PCS can be activated by the presence of toxic metal ions in plant cells [15].

Moreover, we attempted to determine changes in K_m for PCS in tobacco cells treated with cadmium (II) ions (Fig. 3A). The K_m was determined for all concentrations by saturation curve (Fig. 3A) and by Lineweaver–Burk plot (inset of Fig. 3A). Calculated values of K_m did not differ from one another and are shown in Fig. 3B. The highest K_m (2.3 mM) for PCS was measured in non-treated cells. This constant decreased with increasing concentration of cadmium (II) ions except the highest applied concentration at which the increase was observed. This might be associated with the phenomenon that cells trigger certain supporting mechanisms to enhance the main defence mechanisms such as synthesis of PCs. Nakazawa et al. attempted to determine PCS in non treated cells and found K_m of PCS (GSH as a substrate) as 8.6 mM. The results are in good agreement with Nakazawa et al. [41].

4. Conclusion

We showed that HPLC-ED with detection limits as femtomoles of PC_2 can be used for simple and fast determination of activity of phytochelatin synthase. Phytochelatins are currently being intensively studied in lower and higher plants and more recently also have been identified in animals (in the nematode *Caenorhabditis elegans* [15]). Thus, HPLC-ED represents new tool that significantly contributes to the possibilities of analytical chemistry of metal-binding peptides. Moreover, this tool can be used not only for screening of PCS in various plant samples but also for evaluation of plant protective mechanisms against heavy metal ions. Screening tool for determination of PCS opens new possibilities in directing of transgenic modifications as well as can bring new insight into metal-plant cell interactions.

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