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Application of selective extraction and reverse phase chromatography with three detectors – PAD, FLD and ESI MS for characterization of platinum metabolites and identification of phytochelatins in *Sinapis alba* L. tissues

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ABSTRACT

Several extraction procedures, with reagents having the potential to select different groups of compounds (water-soluble, polypeptide and polysaccharide complexes) were used to characterize platinum species in hydroponically cultivated plant white mustard (*Sinapis alba* L.). It was found, that 10% (roots) to 50% (leaves) of platinum species present in plants are soluble in water. Platinum is also bound to the hydrophobic proteins and polysaccharides in the cell walls. In plants exposed to the contaminant during the whole time of their cultivation, platinum was stronger bond to the matrix in comparison with plants exposed to platinum salt for the last week of their growth. To control the synthesis of phytochelatins in plants three different procedures were applied. Triple-step pulsed amperometry was used to detect selectively sulfur-containing compounds after their chromatographic separation. In the extract of mustard leaves together with other investigated thiol-compounds, phytochelatin containing two α-glutamylcysteine dipetide groups (PC_2) was found. The presence of PC_2 in plant extract was confirmed by HPLC FLD and ESI MS analysis after thiols derivatisation.

1. Introduction

Interest in studying platinum biocycles in the environment has increased as a consequence of the common use of cars equipped with catalytic converters. During vehicle operation, mechanical abrasion and deterioration within catalytic converters take place and particles containing platinum group elements (PGE) from the PGE-coated ceramic honeycomb monolith are released. Pt emission of 9 ng to 124 ng per km of the road is calculated and usually increases with the increase of vehicle speed [1]. The highest Pt emission is measured for diesel catalysts – 10–100 folds higher than for gasoline [2]. As a result, an increase of platinum concentration in air [3,4], road dust [5–8], soil [9–12] and plants [13–15] is observed. Emitted platinum species, identified mainly as metallic platinum and platinum oxide, are not mobile and available for plants. Even though in case of road dust the amount of water-soluble platinum compounds does not exceed 10% [16,17] due to the fact that in the environment they undergo many chemical processes, they are converted to some bioavailable forms mainly as chloro and organic complexes [18]. The extractability of platinum from tunnel dust increases from 10 to 52% after 30 days of interaction with soil [19]. It is suggested, that metallic platinum emitted from catalyst surfaces is oxidized in the soil, forming complexes with humic acids [17,19].

There are several works, describing bioavailability of platinum for plants exposed to dissolved platinum salts [20,21] as well as growing on polluted soil [7,13,22], but so far knowledge about platinum metabolism in plants is very narrow. Recently the influence of Pt (IV) on the activity of phytochelatin synthase enzyme, which is involved in the formation of phytochelatins in response to stress in plants, was examined. Experiments were carried out on such species as maize (*Zea mays* L.) and pea (*Pisum sativum* L.) [23], but authors have not identified specific phytochelatins.

The phytochelatins (PCs) are a family of metal-complexing peptides, consisting of 2–11 α-glutamylcysteine dipetide groups, which are synthesized in plant cells in the presence of excess of metals including Cd [24,25], Pb [26], Zn [27], Ag [28] or Hg [29]. Synthesis of phytochelatins is one of the possible mechanisms of heavy metal tolerance and detoxification in plants [30,31].

To determine phytochelatins and their complexes present in plant samples, after chromatographic separation, mass spectrometry with element and molecular ionizations are used [25,32–34]. Detection of sulfur-containing compounds is also possible using electrochemical

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methods like pulsed amperometry [35–37]. When thiol compounds, and among them phytochelatins, are derivatised, also fluorimetry can be applied [33,38].

In our previous works [20,39,40] the ability of the three plant species: Indian mustard (*Sinapis alba* L.), grass (*Lolium perenne*) and Anava maize (*Zea mays* L.) for platinum uptake and accumulation was investigated. It was estimated that the concentration of platinum in particular plant organs as well as the ratio of its total amount in the above ground organs and roots, strongly depends on the concentration of the investigated element in nutrient solution and time of cultivation and platinum exposure. Differences were also observed between particular plant species, so it can be suggested that the accumulation mechanism and metabolism of platinum is different for the investigated plants. Despite the quite high concentration of platinum in the investigated plants (within the range 0,3 mg kg^{-1} in above ground organs up to 470 mg kg^{-1} in roots) no changes in morphology or biomass production were observed. All species show high tolerance towards the presence of platinum salt in nutrient solution. But the highest accumulation factors were observed for white mustard [20].

Carrying on the undertaken investigation, the aim of this work was to examine and characterize platinum compounds accumulated in plant tissues. Different extraction procedures were performed to estimate the amount of water-soluble platinum forms and its complexes with proteins and polysaccharides. Additionally, extracted platinum compounds were characterized based on RP HPLC PAD. Measurements were carried out to analyze the presence of biologically important thiol compounds such as cysteine (Cys), reduced (GSH) and oxidized (GSSG) glutathione. But a special attention was paid to the investigation of phytochelatins that could be synthesized in plant tissue exposed to platinum salts added to nutrient solution. For that purpose phytochelatins, after derivatisation were additionally monitored by HPLC FLD and HPLC ESI MS.

2. Material and methods

2.1. Instrumentation

2.1.1. Determination of total platinum content in plants

For determination of total amount of Pt in plant samples approximately 250 mg of dried plant material and 3 mL of concentrated HNO_3 were placed in PTFE vessels and digested in a microwave system (Ethos 1 Advanced Microwave Digestion System, Milestone, Italy). A three-stage program with a maximum temperature of 200 °C and a maximum microwave power of 1000 W was used (5 min: 20–90 °C; 10 min: 90–170 °C; 30 min: 170–200 °C). The digested samples were transferred to volumetric flasks and diluted to the volume of 25 mL with milli-Q water. The measurements were carried out with an ICP-MS (Elan 6100 DRC ICP MS, Perkin Elmer SCIEX, Canada) after dilution of the samples with water. Platinum was quantified using calibration curve by means of standard solutions from 0.50 to 50 μg L^{-1}. The internal standard was Ir – 10 μg L^{-1}.

2.1.2. HPLC PAD and HPLC FLD

Chromatographic separation before PAD and FLD detection was realized with a Model 1100 HPLC pump (Agilent, Wilmington, DE, UDA) as a delivery system. Injections were made using a Model 7725 injection valve with a proper injection loop (Rheodyne, Cotati, CA, USA). All connections were made of PEEK tubing (0.17 mm id).

For separation Zorbax Eclipse XDB C-18 column set consisting of guard column (4.6 × 250 mm, 5 μm) and analytical column (4.6 × 15 mm, 5 μm) (Agilent Technologies, USA) was utilized. 20 μL injection loop was used.

2.1.2.1. RP HPLC PAD. For separation of the investigated thiol compounds before electrochemical detection as a mobile phase 0.05% trifluoroacetic acid TFA/acetonitrile (99:1, v/v) with a flow rate 1 mL min^{-1} was applied. Mobile phase was ultrasonically degassed.

On-line detection was done with electrochemical analyzer μAutolab, (ECO-CHEMIE BV, The Netherlands) equipped with home-made amperometric cell. The cell comprised a working Au disc electrode (Φ = 1.6 mm), saturated Ag/AgCl reference electrode and an auxiliary Pt electrode.

For triple-step pulsed amperometric detection the following electrode potential-time program was used: detection: E_{det} = 1.4 V, t_{det} = 240 ms (acquisition delay t_{del} = 140 ms), oxidative cleaning: E_{oxd} = 1.6 V; t_{oxd} = 180 ms and regeneration of the bare gold electrode surface: E_{red} = −0.8 V; t_{red} = 500 ms.

2.1.2.2. HPLC FLD. Before fluorimeric determination conditions of separation and detection, optimized during our previous studies [41], were applied. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) and acetonitrile (ACN). The following gradient elution program was used: 0–10 min with 8–12% ACN and 10–40 min with 12–35% ACN. The measurements were carried out with an excitation wavelength of 380 nm and an emission wavelength of 470 nm.

The identification of the extracted compounds was performed by comparing the retention times of the obtained peaks to the retention times of standard compounds. Additionally some chromatograms were recorded after addition of standards to sample solution.

2.1.2.3. HPLC ESI MS. The analysis was performed with the Shimadzu LC system consisted of binary pumps LC20-AD, degasser DGU-20A5, column oven CTO-20AC, autosampler SIL-20AC, connected to 3200 QTRAP Mass spectrometer (Applied Biosystem/MDS SCIEX) via additional Valco valve. The MS system was equipped with electrospray ionization source (ESI) operated in positive-ion mode. ESI conditions were as following: capillary temperature 300 °C, curtain gas at 0.3 MPa, auxiliary gas at 0.3 MPa, ionization mode source voltage 4.0 kV. Nitrogen was used as curtain and auxiliary gas.

Compounds were separated on Luna (Phenomenex) C-18 column (100 × 2.1 mm, 5 μm) with precolumn at 30 °C. 8 mM formic acid (pH 2.8) as eluent A and acetonitrile as eluent B were used. The mobile phase was delivered at 0.2 mL min^{-1} in gradient mode: 0–5 min 8% B, 5–15 min 8–35% B, 15–16 min 35% B, 16–18 min 35–80% B, 18–21 min 80%, 21–22 min 80–8% B. To protect ion source capillary against impurities, unretained fraction (0–2 min) was directed to waste. The analytes were identified by comparing retention time and *m/z* values obtained by MS (linear ion trap) with the mass spectra from standards tested under the same conditions.

2.2. Reagents and standards

Decomposition of the plant material was carried out with 65% HNO_3, (Suprapur, Merck). Ammonium acetate and Tris (POCh Gliwice, Poland) were used for buffer preparation. TFA (>99%, Fluka), acetonitrile (Cheman, Poland) and formic acid were utilized as components of mobile phases in RP HPLC. Sodium dodecyl sulfate, the enzymes: protease (type XIV from *Streptomyces griseus*) and driselase (from *Basidiomycetes*) were applied for extraction. The identification of the compounds was performed by comparing the retention times of the obtained peaks to the retention times of standard compounds: cysteine (purity > 97%, Sigma-Aldrich), glutathione (purity > 99%, Sigma-Aldrich), *N*-acetylcysteine (purity > 99%, Sigma-Aldrich) and three phytochelatins: PC_2, PC_3 and PC_4 (purity > 95%, AnaSpec).

To prevent oxidation of —SH groups DDT (Sigma-Aldrich) was added to the solution of PCn.

2.3. Samples

Indian mustard (*Sinapis alba* L.), plants were cultivated in growth chamber, at temperature 20 °C during days and 16 °C during nights