Identification of the binding forms of cadmium during accumulation by water hyacinth

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ABSTRACT

It is well known that the plants are capable to hyperaccumulation but despite their extensive use for water cleaning in environment, the essence of this phenomenon remains unclear. This paper is focused on the development of methodology for the determination of peptides binding forms of the metal in plants on the example of cadmium and water hyacinth. The suggested approach is based on the analytes extraction followed by their HPLC separation with UV and element-selective detection, and at last amino acids and sulfide groups determination in the isolated fractions containing cadmium, and for this, the optimal parameters for the interfacing of microcolumn chromatograph and ICP-OES spectrometer have been developed. It has been shown as a result that cadmium-containing compounds can be attributed to peptides similar to phytochelatins. This approach provides an information concerning the nature of the compounds responsible for the act of detoxification during bioaccumulation process.

Introduction

It is known that phytoremediation technology became an effective method of environment clearing after it has been found the plant’s ability to accumulate the contaminants at the concentration level is thousand times higher than background. In worldwide practice of waste waters purification, the floating macrophytes are applied most often.

In published works devoted to the study of the uptake of the elements by the living organisms,[1,2] the separate stages of interaction between the pollutant and accumulating system are mostly considered. However, a deep understanding of bioaccumulation mechanism for subsequent impact on the process requires knowledge about the binding forms of the elements inside the plant, as it determines the toxicity, mobility, bioavailability, biological activity and the ways of the elements transformation and transport as well. Thus, the identification of the chemical forms of the elements is an actual problem of modern analytical chemistry, important for environmental sciences, as well as geology, clinical and medicinal chemistry.

There are several mechanisms of plant response to exposure to toxic agents including heavy metals, which are discussed in the literature. In most cases, this reaction consists in immobilization, the formation of chelate complexes, depositing and at last removal of metal ions from the living organism. This process may also involve the specific mechanisms such as the activation of oxidative stress-related enzymes (catalase, peroxidase and superoxide dismutase), the change of physicochemical properties of the cell membranes and the changes in hormonal balance. As the most common detoxification mechanism, the formation of the metal’s complex compounds accompanied by their depositing into the plant cell organelles is often considered. Understanding of the molecular and genetic basis of these mechanisms may allow more efficient use of the certain plants as agents for the contaminated areas phytoremediation.[3,4]

Water hyacinth (Eichhornia crassipes, EC), as a hyperaccumulator,[5] attracts more and more attention of researchers. This plant is applied most often to sewage treatment of the industrial enterprises [6,7] and may consider as efficient tool for contaminated waters cleaning up resulting in sustainable as well as energy and cost economic process.[8] Despite the growing interest in the practical application of this plant for waters cleaning, there are only a few studies on the binding of accumulating elements with biologically active molecules. Thus, Fujita Masayuki [9] discussed an ability of EC to synthesize metallothioneins – the proteins of the molecular weight of 2300–3000 Da with high cysteine content in response to cadmium impact. At the same time, there is an evidence of the binding of cadmium at the initial stage of accumulation with the compounds...
of non-protein nature,[10] and at a longer exposure – metal depositing in plant’s root tissues as a complex with peptides, which are the products of enzymatic synthesis – phytochelatins with the polymerization degree of 3–4. Cadmium compounds of protein nature relating to phytochelatins were also found in the leaves while some of them have not been identified.[11]

In the present work, the authors suggested an approach for cadmium species identification in EC forming during bioaccumulation process. Within this approach, the procedure of protein compounds extraction from the plant tissues was modified, the fractionation of proteins using HPLC and identification of cadmium-containing fractions by HPLC-ICP-OES was performed and, at last, an amino acid composition as well as thiol-groups content in these fractions was determined.

**Methodology**

**Reagents**

Cadmium nitrate, Cd(NO₃)₂, was added as pollutant into the container with the plant; nitric acid, HNO₃; hydrochloric acid, HCl; and boric acid, H₃BO₃; were used as the reagents in plants’ digestion procedure. All reagents were not worse than reagent grade (RG) and were purchased in Reachim (Russia, Moscow). Certified multielemental mixture of Ltd. Scat (Russia, Novosibirsk) was employed as reference samples in ICP-OES analysis. Acetonitrile, CH₃CN; acetic acid, CH₃COOH; ammonium acetate, CH₃COONH₄; sodium acetate, CH₃COONa (all reagents were HPLC grade); TBAG, 10% solution; were used for the preparation of the mobile phases in HPLC. Phenyl isothiocyanate (PITC, Fluka), sodium carbonate, and individual amino acids (Sigma-Aldrich) were used for amino acid analysis; Tris HCl, sodium hydrogen phosphate, and sodium dihydrogen phosphate were applied as the components of the buffers in extraction procedures.

**Instrumentation**

HPLC system Milichrom A-02 (Econova, Russia) with micro column ProntoSIL-120-5-C18 AQ was used for the separation of cadmium-containing fractions and amino acids determination in them. ICP-OES spectrometer iCap 6500 Duo (Thermo Scientific, USA) with standard concentric nebulizer was applied for the elements determination in plants and as element-selective detector for HPLC. The data acquiescing and processing was provided by iTEVA (Thermo Scientific, USA) software. Plant tissues digestion procedure for the trace elements determination by ICP-OES was performed using Mars-5 (CEM, USA) system. Determination of thio-groups in plant’s extract was carried out by stripping voltammetry (IVA-5, Russia) using three-electrode electrochemical cell with working platinum electrode (Radelkis Electrochemicals Instruments, Hungary), an auxiliary electrode of glassy carbon or graphite rod and the silver chloride (Ag/AgCl) as a reference one.

**Laboratory experiment**

The scheme of the experiment is presented in Table 1. After 1, 3, and 7 days of the experiment, the plants were removed from the containers successively, weighted, divided into the parts, and dried. Then, the tissues were exposed to a mechanical mill and subjected to microwave-assisted digestion for subsequent determination of cadmium content. Additionally, the parts of fresh root, stem, and leaf were frozen in liquid nitrogen for the following extraction and identification of cadmium compounds with biologically active molecules.

**Experimental approaches and methods**

The compounds which are capable to bind cadmium ions in plant tissues include peptides with a high content of sulfhydryl groups, organic acids, and pectin as the components of the cell wall.

According to the published data concerning these species determination, the results obtained by different researchers are often not in agreement with each other.[9,10] In some cases, the preference is given to the protein nature compounds – metallothioneins,[9] whereas phytochelatins which are the products of the enzymatic synthesis are most often considered as chelators in plants.[10,11]

To identify the main cadmium binding forms in water hyacinth, we proposed the methodology, the essence of which is illustrated by the scheme (Figure 1).

**Trace elements determination in water and plant samples**

Sample preparation procedure for the elements determination by ICP-OES in plant’s tissues included a digestion procedure in the presence of the reactant’s mixture containing 5 mL of HNO₃, 2 mL of HCl, and 0.4 mL of HF per 0.10 g of plant tissue using microwave-assisted system according to the program: (1) 10-min ramping to 90 °C, 10-min holding at 90 °C; (2) 10-min ramping...
to 150 °C, 10-min holding at 150 °C; (3) 10-min ramping to 180 °C, 10-min holding at 180 °C. After completion of the mineralization procedure, the samples were quantitatively transferred to the tubes, bringing the volume to 10 mL with deionized water where 2.5 mL of 4% boric acid was added to eliminate the influence of fluoride ions by reaction: \( \text{H}_3\text{BO}_3 + 4\text{F}^- \rightarrow \text{[BF}_4^- + 3\text{OH}^- \). Before analysis, scandium nitrate at the level of 0.5 mg L \(^{-1}\) was added to the probe as internal standard for ICP-OES analysis according to the following working parameters: power supply, 1150 W; argon flows, L min \(^{-1}\): 0.7 for the nebulizer, auxiliary – 0.5, cooling – 12. Linear dynamic range of the calibration curve for cadmium and sulfur was 10\(^5\) (0.001–100 mg L \(^{-1}\)).

**Cadmium compounds isolation from the plant**

To retrieve the cadmium–protein compounds from the plant’s tissues (roots, stems, and leaves), the following procedure was applied as follows: 0.100 g of frozen matter was crushed, homogenized in a quartz mortar on the ice with 1 mL of extraction buffer, held during 30 min and centrifuged (30 min at 18,000 rev/min at 4 °C). Supernatant was then filtered through a membrane filter (0.45 μm) and stored at −20 °C. An extraction medium was chosen relied on the published data [12] with a slight modification by the use of ultrasonic accelerating of the process. Taking as a criterion a maximum of cadmium extraction (Table 2), deionized water was selected as extraction medium. According to Kamnev [13], the peptide compounds which are able to bind with metals do not exceed 10–15 kDa and therefore are water soluble. The use of ultrasound has resulted in cadmium extraction at the level of 9–10% during 15 min.

The protein-containing extracts were then separated into the fractions by molecular weight using HPLC in accordance with [12] Two modes of HPLC combination with ICP-OES have been used: online which allowed the identification of cadmium-containing fractions and offline which permitted to isolate the desirable fractions for further investigation. In conformity with the published data, we can assume that these fractions contain compounds of polypeptide nature. To test this hypothesis and identify the water-soluble cadmium compounds which are present in plant’s tissues, the isolated materials were subjected to acidic hydrolysis followed by amino acid specification and thiol-groups determination.

**Plant’s extracts separation using HPLC-ICP-OES**

**Optimization of the separation conditions**

The separation of cadmium peptide-containing compounds using hyphenated reversed phase ion pair HPLC (RP-IP-HPLC)-ICP-OES in conformity with [12] has been hampered by the problem of plasma quenching thru the high content of acetonitrile in mobile phase. Consequently, the composition of the latter was chosen experimentally (Table 3).

**The parameters of HPLC-ICP-OES offline mode**

To collect the fractions containing the compounds of interest for further examination, the extract was subjected to fractionation under the same conditions as in online mode. For this purpose, the sequential volumes of 0.1 mL were collected at the outlet of HPLC-UV cell to determine the cadmium and sulfur content according to the procedure presented earlier. To confirm the presence of proteins in the isolated compounds, they were hydrolyzed for subsequent amino acids determination. The fractions with the same number from a few runs were combined to provide an adequate volume of the sample to be analyzed at the level of 1.0 mL.

**Amino acids determination in cadmium-containing fractions**

The fractions isolated by chromatographic separation of the plant’s extract were hydrolyzed, subjected to derivatization with phenyl isothiocyanate, and then to HPLC

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**Table 2. An effectiveness of cadmium extraction from the plants under different conditions.**

| Extractant                        | Cadmium recovery (%) |
|-----------------------------------|----------------------|
| Deionized water, ultrasonic assisted extraction | 9–10                |
| Deionized water                   | 5–7                  |
| 200 mM phosphate buffer, pH 7.2   | 4–6                  |
| 10 mM Tris HCl, pH 8.0            | 0.5–1.5              |

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**Figure 1.** The scheme of identification of cadmium binding forms.
Sulfur actively participates in the metabolism of cadmium compounds in the body of the plant. Entering the plant tissue in the form of sulfate, the compounds containing sulfur are quickly reduced and have part in the synthesis of glutathione and phytochelatins. It is known that the synthesis of mRNA encoding proteins involved in sulfate transport and phytochelatins formation is intensified in the plants with a high resistance to heavy metals.[21]

As seen in Figure 3, the most part of cadmium after 7 days of exposure is concentrated in roots (83%), while the least – in leaves (4%). Withal the roots and the stems of the plant contains 0.16 ± 0.03% and the leaves 0.11 ± 0.02% of sulfur which agrees with the published data on the average sulfur content in plant’s organisms (~0.2%).[21] The extracts of the plants under investigation comprise (89 ± 18), (78 ± 16), and (72 ± 15) mg sulfur per kg of fresh weight in roots, stems, and leaves, respectively. Since sulfur recovery in the process of extraction is 55–67% with respect to concentration in plant, it may be concluded that in plant tissues, sulfur predominantly exists as hydrophilic compounds.

Thiol-groups determination in cadmium-containing fractions

Determination of thiol-groups in plant’s extract was carried out using the stripping voltammetric titration method based on the deposition of organic compounds containing SH-groups according to reaction: $\text{RSH} + \text{AgNO}_3 \rightarrow \text{AgSR} + \text{HNO}_3.[17]$

Results and discussion

Elements distribution between the parts of the plant

The results of the study of cadmium transport inside the plant are presented in Figure 2. It is seen that cadmium concentration in plant roots increases sharply reaching the saturation level within 24 h, wherein it’s transport into the stem intensifies. In leaves, cadmium concentration varies slightly during a day as it was shown earlier [18] but at longer times it also increases. We suggested that at the first stage of bioaccumulation process, the sorption of the pollutant on the surface of the roots takes place. To the extent of penetration into the plant cadmium is mainly localized in the cortex and rhizodermis of the roots.[18] According to Viehweger [19], activity of metal-sequestering pathways in root cells plays a key role in determining the rate of translocation to the stems and leaves of the plant. A second factor is the degree of accessibility and mobilization of sequestered metal. Also a fraction of the vacuolar Cd bound in the high molecular weight complexes can apparently be mobilized back into the cytosol by some proteins. Loading of the xylem is dependent on efflux pumps residing in the plasma membrane of surrounding cells. An efflux of Cd(II) complexes with phytochelatins has also been proposed.[20]
Identification of cadmium compounds in plant’s extracts by HPLC-ICP-OES

The main areas of cadmium localization on chromatogram of the plant’s extract were established using hyphenated HPLC-ICP-OES with sequential UV and element-selective detection. It was found that analytical signals of cadmium are registered on chromatogram of the root’s extracts but practically absent in extracts of stems and leaves, which may be associated with the element content lower than detection limit (0.01 μg L\(^{-1}\)). The obtained information was used for subsequent isolation of cadmium-containing compounds to determine cadmium, sulfur, and amino acids composition. The results for the roots of the plant exposed to cadmium are shown in Figure 4. Two peaks are registered on chromatogram (Figure 4(a)): the first one at 0.5 min corresponds to restrained compounds apparently none related to cadmium, and the second (3.5 min) coincides with cadmium localization zone.

Further, the consistent isolation of cadmium-containing fractions followed by the quantification of the test element and its binding forms identification was performed. The chromatogram of the roots extract in offline mode of ICP-OES is shown in Figure 4(b), in which the total quantity of cadmium in roots extract calculated from the data on its content in each fraction coincides with its amount injected into the column (for roots from container 7 9.5 ± 2.0 ng – injected, 9.7 ± 2.0 ng – total Cd content in fractions; for roots from container 9 5.0 ± 1.0 ng and 6.9 ± 1.4 ng, respectively), indicating that the monitoring element is almost entirely concentrated in the peak with the retention time of 3.5 min.

The evaluation of thiol-groups function in cadmium binding inside the plant

According to the published data, there are different classes of compounds which can participate in the binding of heavy metals in plants: organic acids, sulfur-containing peptides, and flavonoids. To determine thiol-groups in isolated fractions containing cadmium, we had to use the method which allows obtaining rapid information on their content because of oxidation in the presence of atmospheric oxygen, therefore a stripping voltammetry method was applied. Determination of SH-groups in fractions corresponding to cadmium localization permits estimating what portion of sulfur is involved in the binding of the metal, and comparing the results with the data on the total amount of the element. It has been found that SH-groups distribution in these fractions correlates with those for cadmium, which may be considered as an argument in favor of the binding of the metal with thiol-groups (Figure 5). Furthermore, the total amount of sulfur introduced into HPLC column is the same as a sum of its contents in cadmium-containing fraction. The molar ratio of thiol-groups to cadmium is close to 40. So, we can conclude that ions of cadmium are bound either with the big molecules with a large number of thiol-groups like metallothionein or with the smaller ones such as phytochelatins which are present in excess.

Amino acid composition of cadmium compounds with peptides

To determine the nature of cadmium compounds formed in water hyacinth during bioaccumulation process, the possibility of formation of two main types of compounds with a high content of thiol-groups was taken into account. So, Fujita Masayuki [9] discussed...
Table 4. Amino acid composition of cadmium-containing fractions.

| Amino acid (μmol L⁻¹) | Roots 7 (Cd) | Roots 8 (Cd) | Roots, control | Stems 7 (Cd) |
|-----------------------|--------------|--------------|---------------|--------------|
| C Glu                 | 1.4 ± 0.3    | 1.0 ± 0.2    | 7.2 ± 1.4     | 0.3 ± 0.1    |
| C Cys                 | 0.5 ± 0.2    | 1.4 ± 0.4    | 3.9 ± 1.0     | 0.2 ± 0.1    |
| C Gly                 | 3.0 ± 0.6    | 2.5 ± 0.5    | 9.1 ± 1.8     | 0.4 ± 0.1    |

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Disclosure statement

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