Talanta 77 (2009) 1830–1836

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

# Evaluation of the arsenic binding capacity of plant proteins under conditions of protein extraction for gel electrophoretic analysis

Anne-Christine Schmidt*, Sandra Steier, Matthias Otto

*TU Bergakademie Freiberg, Faculty of Chemistry and Physics, Institute of Analytical Chemistry, Leipziger Str. 29, D-09596 Freiberg, Germany*

ARTICLE INFO

*Article history:*
Received 17 July 2008
Received in revised form 14 October 2008
Accepted 17 October 2008
Available online 31 October 2008

*Keywords:*
Protein extraction
Plant samples
Arsenic-binding proteins
Gel electrophoresis
Atomic spectrometry

ABSTRACT

As prerequisite for the investigation of arsenic-binding proteins in plants, the general influence of different extraction parameters on the binding behaviour of arsenic to the plant protein pool was investigated. The concentration of the extraction buffer affected the extraction yield both for proteins and for arsenic revealing an optimal buffer concentration of 5 mM Tris/HCl, pH 8. The addition of 1 or 2% (w/v) SDS to the extraction buffer produced a two- to threefold enhancement of the total protein extraction yield but strongly suppressed the simultaneous extraction of arsenic from 80 ± 8% extraction yield obtained without SDS to 48 ± 2% in presence of 2% (w/v) SDS. The arsenic binding capacity of the protein fraction obtained after extraction with Tris buffer and protein precipitation by trichloroacetic acid in acetone was estimated to be 1.4 ± 0.6% independently on the original spiking concentration of arsenic provided in the form of monomethylarsonate to the extracts. Due to the low total protein concentrations of the plant extracts that varied in the range from 75 to 412 μg $mL^{-1}$ depending on the extraction parameters, high arsenic concentrations of 263–1001 mg (kg protein mass)$^{-1}$ resulted for spiking concentrations of 10 mg As $L^{-1}$. The optimized protein isolation procedure was applied to plants grown under arsenic exposure and revealed a similar arsenic binding capacity as for the spiked protein extracts.



## 1. Introduction

Toxic effects of arsenic are ascribed to its interaction with biochemical pathways, enzymes, and other biomolecules. In plants, arsenic is taken up from contaminated soils and waters via the roots is further translocated to shoots and leaves [1]. Whereas some plant species are damaged, other kinds of plants show an elevated arsenic tolerance and hyperaccumulate this toxic metalloid [2,3]. Analytical approaches are mainly focused on soluble arsenic species [1,4]. Their possible bindings to biomolecules dissociate during extraction or chromatographic separation. The binding of arsenic to the tripeptide glutathione and to special thiol-rich polypeptides called phytochelatins was demonstrated in plant extracts [5–7] but their analysis is difficult due to limited stability. Arsenic-bindings to high molecular weight compounds such as proteins were found in model solutions [8,9]. However, owing to the fact that many plant enzymes have free thiol groups which can react with arsenic, studies concerning the analysis of arsenic-binding proteins in real plant samples are missing probably due to the difficulty in sample preparation and conservation of the bindings. A first approach concerning this topic was performed by the analysis of the distribution of arsenic species between the fractions of soluble proteins, structure proteins, and non-proteins of arsenic-accumulating plants by means of a fractionated sample preparation procedure [10].

One- and two-dimensional gel electrophoresis are a powerful tool for the analysis of protein patterns of biological samples containing a complex mixture of differently sized proteins. Moreover, a subsequent determination of metals or other heteroelements in the separated protein bands by means of laser ablation inductively coupled plasma mass spectrometry (LA-ICPMS) or X-ray spectroscopic techniques provide the possibility to detect metal- or metalloid–protein complexes [11,12]. Because sample preparation is considered to be the most crucial step for protein analysis by gel electrophoretic separations and very little is known about its influence on the stability of arsenic–protein bindings, it is necessary to investigate the effects of these various parameters.

In contrast to animal or bacterial samples, plant tissues represent a challenging sample type for protein isolation and analysis because they contain plenty of substances such as phenolic compounds, carbohydrates, organic acids, lipids, and terpenes that interfere with the gel electrophoretic separation of proteins [13,14]. Therefore, purification steps such as protein precipitation are necessary to remove non-protein contaminants. Furthermore, protein precipitation serves as a very effective preconcentration step for proteins from diluted protein sources such as plant materials. Because trichloroacetic acid (TCA) in acetone has been proven as

* Corresponding author. Tel.: +49 3731 393255; fax: +49 3731 393666.
*E-mail address:* Anne-Christine.Schmidt@chemie.tu-freiberg.de (A.-C. Schmidt).

0039-9140/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2008.10.027

an efficient precipitating agent for purification of plant proteins for subsequent gel electrophoretic separation [15–17] this type of precipitation was also applied in the current study. A further advantage of the TCA/acetone precipitation consists in the immediate inactivation of proteolytic and other modifying enzymes that are released from broken cells during extraction [18]. However, in some cases a washing of the protein precipitate is necessary prior to gel electrophoresis to remove co-precipitated contaminants [14].

To search for real arsenoproteins in biological samples, the exploration of the behaviour of arsenic during extraction, precipitation, and purification of proteins for gel electrophoresis becomes obligatory. In the present investigations, the arsenic binding capacity of plant proteins extracted under different conditions was determined exemplary of a biologically important arsenic compound, monomethylarsonic acid (MMA(V)). This methylated arsenic species is assumed to be produced from inorganic arsenite during biomethylation and was also found in plants grown on arsenic-enriched substrates [19,20]. Further, due to their strong toxicity, monomethylarsonate salts were used as herbicides [21]. The influences of different parameters varied during sample preparation on the extraction efficiencies obtained both for total arsenic and for total protein as well as on gel electrophoretic separations of the extracted plant proteins were evaluated. After optimization of the sample preparation procedure by means of arsenic spiking experiments, the optimized extraction parameters were applied to plants cultivated under arsenic exposure in order to quantify the fraction of accumulated arsenic that was associated to proteins.

## 2. Experimental

### 2.1. Plant material

Leaf and stalk material from simply cultivable nasturtium plants (*Tropeaolum majus* L.) served as protein source for the preparation of protein extracts. In addition to control plants grown on humus soil characterized by a background arsenic level of <5 mg $kg^{-1}$ dry mass, some plants were cultivated under arsenic exposure during 8 weeks after an initial arsenic-free germination and growth phase of 4 weeks. The arsenic compound monomethylarsonate (MMA(V), obtained in the form of monosodium acid methane arsonate sesquihydrate from Chem Service, West Chester, PA, USA) was applied to the soil used for plant cultivation twice per week in 100 mL portions containing 5 mg arsenic. The plants were harvested and divided into leaves and stalks, and stored frozen at −20 °C.

### 2.2. Preparation of the extraction media

The solid buffer substances tris(hydroxymethyl)aminomethane (Tris, p.a., from Merck, Darmstadt, Germany) and ammonium acetate (HPLC grade, from J.T. Baker B.V., Deventer, Holland) used for the extraction experiments were dissolved in deionized water to prepare 100 mM buffer stock solutions. To vary the pH for the extraction media, the pH value of the buffer solutions was adjusted with 2 M HCl (prepared from 32% HCl, p.a., Sigma–Aldrich, Steinheim, Germany) or with 100% acetic acid (p.a., Merck, Darmstadt, Germany), respectively, and controlled by a pH electrode (HI 9321 microprocessor pH meter, HANNA instruments, Kehl am Rhein, Germany). Different buffer concentrations were prepared by dilution of these stock solutions with deionized water.

The reducing agent dithiothreitol (DTT, 1 M stock solution in water from Sigma–Aldrich) was added to the extraction buffer system to a final concentration of 50 mM. Further extraction buffer additives were the protease inhibitor phenylmethylsulfonyl fluoride (PMSF, 0.1 M stock solution in ethanol from Sigma–Aldrich) at a concentration of 2 mM in the final extracts and 10 mg of the adsorbens for polyphenolic compounds polyvinylpolypyrrolidone (PVPP, from Sigma–Aldrich) in an extract volume of 10 mL. The concentration of the tenside sodium dodecyl sulfate (SDS, ≥98% from Sigma–Aldrich) in the extraction buffer was varied between 0, 0.5, 1, and 2% (w/v).

### 2.3. Combined protein and arsenic extraction protocol for plant tissues

The flow-chart of the entire extraction and purification protocol is pointed out in Fig. 1. The extractions were performed in 30 mL polypropylene centrifuge tubes (Nalge Nunc International Corporation, Rochester, NY, USA). For the centrifugation steps the AvantiTM Centrifuge J-30I (Beckmann Coulter, Fullerton, CA, USA) was used. The ultrasound treatments were carried out in a water bath. For some samples, the final protein pellet was washed with 4 mL 90% (v/v) ethanol containing 2 mM DTT for 2 h, and centrifuged again at 10,000 × *g* and 4 °C for 10 min. The precipitating agent acetone was purchased in p.a. quality from Merck, Darmstadt, Germany, and trichloroacetic acid (TCA, ≥99.5%) from Sigma–Aldrich, Steinheim, Germany.

### 2.4. Microwave digestion of extraction residues and protein pellets

The filtration and centrifugation residues arising from the extraction procedure as well as the precipitated protein pellets (Fig. 1) were mineralized by means of microwave digestion with 3 mL 65% $HNO_3$ and 1 mL 30% $H_2O_2$ (both in suprapur quality from Merck, Darmstadt, Germany) in 20 mL Teflon vessels using the following program (Microwave system Start 1500, MLS, Leutkirch, Germany): step 1: 3 min heating to 85 °C at a power of 700 W; step 2: 5 min heating to 145 °C at a power of 1000 W; step 3: 3 min heating to 210 °C at 1000 W; step 4: 15 min maintaining the temperature at 210 °C and the power at 1000 W; step 5: cooling to 20 °C for 20 min at 0 W. The resulting digests were filled up to 8 mL with deionized water. Three blank samples containing 3 mL $HNO_3$ and 1 mL $H_2O_2$ were processed as the other samples.

### 2.5. Protein quantification by the Bradford assay

For preparation of the Bradford reagent, 50.7 mg Coomassie Brilliant Blue G-250 (from Sigma–Aldrich) were dissolved in a mixture of 25 mL 95% (v/v) ethanol (p.a., from Merck) and 50 mL 85% (w/v) orthophosphoric acid (ultra, Sigma–Aldrich). This solution was filled up with deionized water to a final volume of 500 mL and filtrated to remove insoluble dye particles. 200 μL of the dye reagent solution were mixed with 800 μL aqueous sample solution and incubated for 10 min. A calibration line was compiled by measuring the UV absorption at 595 nm for six different concentrations of bovine serum albumine (BSA, 200 mg $mL^{-1}$ stock solution in water from Sigma–Aldrich) using a photometer (Ultrospec 1000, Pharmacia Biotech). The linear range was estimated to 0.1 to 4.0 μg $mL^{-1}$ ($r$ = 0.984). The leaf protein extracts were diluted 1:100, 1:50, or 1:10 with water in order to produce absorption values that were consistent with this calibration range. The absorption value of a mixture of 200 μL dye reagent and 800 μL deionized water was subtracted as blank value from all BSA and sample values.

### 2.6. ICP-OES and HG–ETA-AAS for determination of total arsenic contents

The total arsenic contents in the raw protein extracts as well as in the microwave digests of the extraction residues and of the