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Sample preparation strategies for one- and two-dimensional gel electrophoretic separation of plant proteins and the influence on arsenic and zinc bindings

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ABSTRACT

A sample preparation method including protein extraction by an aqueous buffer system, precipitation with trichloroacetic acid, washing with acetone, and desalting by dialysis was developed for 2D gel electrophoresis of mature leaves from *Tropaeolum majus*, a plant species with a high content of glucosinolates. By the optimized method, 1D- and 2D-gels could also be produced from *Festuca rubra* leaves and *Helianthus annuus* seeds. A strong influence of the varied protein preparation parameters on arsenic and zinc bindings was observed. Microwave-digestion with subsequent atomic spectroscopy analysis of protein fractions revealed the highest arsenic binding capacity of 76.2 ± 1.7% for proteins from sunflower seeds spiked with arsenite. After spiking of *T. majus* extracts with different arsenic species and zinc salts to 100 μg As or Zn in 10 mL, 9.5 ± 0.97%, 0.95 ± 0.39%, 0.24 ± 0.02%, 0.20 ± 0.09%, 0.02%, 0.83 ± 0.02%, 2.21 ± 1.64%, and 1.45 ± 0.69% were recovered in the final protein fraction for phenylarsine oxide, arsenite, arsenate, monomethylarsonate, dimethylarsinate, zinc chloride, zinc sulfate, and zinc acetate, respectively. The cultivation of *T. majus* under arsenic exposure resulted in a highly elevated arsenic-binding capacity of the proteins that was also dependent on the kind of arsenic species in the following order: arsenite (14.9%) > monomethylarsonate (12.4%) > arsenate (10.8%) > dimethylarsinate (0.32%).

1. Introduction

Plants can accumulate toxic arsenic compounds from soil and water [1]. Under the influence of arsenic or other metal(oid)s, the protein expression is regulated to synthesize metal- and metaloid-binding proteins such as metalothioneins [2], phytochelatins [3], heat shock proteins [4], and cyclophilines [5] for detoxification. Otherwise, the protein synthesis can be negatively affected due to toxic effects like enzyme inhibition. Even though a lot of studies exist about the speciation of low molar mass arsenic compounds in plants [6,7], the detection of arsenic bindings to biomolecules in plant samples hardly succeeded as yet [8]. From mass spectrometric studies of model solutions, arsenic bindings to thiol-containing peptides and proteins have been deduced [9]. Currently, arsenic-binding proteins were identified in human cells by liquid chromatography coupled to mass spectrometry [10].

For the studies of the protein patterns of plant tissues, the two-dimensional gel electrophoresis (2D-GE) provides a high resolution [11,12] and offers the possibility to detect metal- or metaloid-bindings to the separated protein spots by means of laser ablation and inductively coupled plasma mass spectrometry (LA–ICPMS) [13]. So far, only a few studies about the proteomic analysis of arsenic-contaminated plants can be found in the literature [14]. Especially for plants, the extraction and purification of proteins for a 2D-GE analysis is of high demand because plant cells are characterized by a low protein content and a high amount of interfering substances [15,16]. The majority of protein extraction protocols has been optimized for tissues of seedlings or young plants. However, effects of pollutant expositions to plants should be observed over a longer growth period. In a study of Islam et al. [17] the challenges for the extraction and purification of proteins in matured leaves are pointed out.

For the analysis of metal- or metaloid-binding proteins by 2D-GE, the stability of the bindings during sample preparation must be considered. Systematic investigations about the extractability of metal(oid)-binding proteins from biological tissues are rare. For example, ultrasound treatment improved the total protein extraction yield but destroyed bindings of Cr and Mn of plant proteins extracted with water or buffer [18]. In a previous work, a sample preparation protocol for the analysis of plant proteins by one-dimensional gel electrophoresis (1D-GE) was established for nasturtium plants cultivated under arsenic exposure [19]. For 2D-GE separations a greater effort is required in the protein sample handling than for 1D-GE since the first dimension consisting in the isoelectric focusing step is substantially more susceptible to interferences. Based on the results obtained in [19] for 1D-GE approaches, the optimization of protein extraction and purification from matured leaves of *Tropaeolum majus* for subsequent 2D-GE

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analysis is presented in the current work. The influence of the varied sample preparation parameters on arsenic and zinc bindings to the protein fraction was evaluated. *T. majus* was chosen for these investigations since accumulation and metabolization of inorganic and organic arsenic species by this kind of plant were elucidated previously [20]. The sample preparation scheme was also applied to other types of plant sample matrices such as sunflower seeds and grass to demonstrate the capability of the method. Finally, the arsenic-binding capacity of the protein fraction from plants cultivated under exposure to different arsenic species was determined.

2. Materials and methods

2.1. Plant cultivation

After a 4-week germination and growth phase of *T. majus* in pots filled with humus soil without arsenic application, 100 mg arsenic in the form of monomethylarsonate (MMA(V)), dimethylarsinate (DMA(V)), arsenite (As(III)), or arsenate (As(V)) were provided in aqueous solutions to the soils in a period of 8 weeks in 6.25 mg portions twice per week. As(III) was applied in a 2nd series to 50 mg As over 4 weeks. MMA(V) was purchased as monosodium acid methane arsonate sesquihydrate from Chem Service (West Chester, PA, USA), DMA(V) as sodium cacodylate trihydrate (≥98%), As(III) as 0.05 M sodium arsenite solution, and As(V) as disodium hydrogen arsenate heptahydrate (≥98%) from Sigma–Aldrich (Steinheim, Germany). The plants used for 2D-GE method optimization purposes were cultivated in humus soil without arsenic contamination. The leaves were harvested and stored at −20 °C until protein preparation for 1- and 2D-GE. Dried and peeled sunflower seeds (Kluth, Nature Garden, Henstedt-Ulzburg, Germany) were purchased in a food market. Aboveground parts of red fescue (*Festuca rubra* L.) were harvested from a meadow situated in Freiberg, Saxony, in June 2008, and processed immediately.

2.2. Protein preparation procedure

All included steps except for heat precipitation were carried out on ice. The sample solutions were processed in 30 mL polypropylene centrifuge tubes (Nalge Nunc International Corporation, Rochester, NY, USA). The centrifugations were performed in the AvantiTM Centrifuge J-30I (Beckmann Coulter, Fullerton, CA, USA).

The protein preparation procedure for 2D-GE analysis (Fig. 1) was divided into five successive steps: (i) protein extraction from leaf tissue, (ii) protein precipitation, (iii) washing of the protein precipitate, (iv) resuspension of the precipitated protein, (v) desalting of the final protein solution. The chemicals for the extraction buffer optimized in our previous work [19] were from Merck, Darmstadt, Germany (tris(hydroxymethyl)aminomethane, tris, p.a.) or from Sigma–Aldrich (hydrochloric acid, 32%, p.a., phenylmethylsulfonyl fluoride, PMSF, 0.1 M stock solution in ethanol, polyvinylpolypyrrolidone, PVPP). The phenolic extractant (phenol, ultra, Sigma–Aldrich) was prepared according to Ref. [21]. A reducing agent level of 2 mM tris(carboxyethyl)phosphine (TCEP, 0.5 M, Sigma–Aldrich) was maintained throughout the entire sample preparation procedure to prevent protein oxidation and aggregation. Arsenic and zinc were added in the form of MMA(V), DMA(V), As(V), As(III), or phenylarsine oxide (PAO, ≥97%, Sigma–Aldrich), or of different zinc salts (acetate dihydrate, ≥99.5%, chloride, ≥98.0%, sulfate heptahydrate, ≥99.0%, all from Sigma–Aldrich), to the raw protein extract to a concentration of 10 mg L^{-1}. Protein concentrations in the extracts were quantified by the Bradford assay as described in Ref. [19]. Calibration solutions with bovine serum albumin (200 mg mL^{-1} stock solution in water from Sigma–Aldrich) were prepared in the extraction buffer system consisting of 5 mM tris adjusted to pH 8.0 with 0.2 M HCl, 2 mM PMSF, 2 mM TCEP.

The protein-containing extract was mixed with different precipitating agents: 20% (m/v) trichloroacetic acid (TCA, ≥99.5% from Sigma–Aldrich) in deionized water or in acetone (p.a., from Merck) in a volume ratio of 1:1, or pure acetone in a 1:4 ratio of sample to solvent. The heat precipitation was carried out in a water bath. The precipitates were washed in one, two or three cycles with 2 mL 2 mM TCEP containing acetone, methanol (HPLC grade, Fisher Scientific, Leicestershire, UK), or ethanol (p.a., Merck, Darmstadt, Germany).

Before IEF, the resolubilized proteins were desalted using dialysis tubes with a cut-off value of 1 kDa and 2 mL maximal sample volume (Mini Dialysis Kit, Amersham Biosciences, USA) against 30 mL 8 M urea (electrophoresis grade, Sigma–Aldrich) solution by stirring for 18 h at 4 °C. The urea solution was regenerated after 4 h. Alternatively, PD-10 desalting columns packed with Sephadex™ G-25 (GE Healthcare) were equilibrated six times with 4 mL 8 M urea solution. The proteins were eluted with rehydration solution (composition in Section 2.3). Desalted samples were stored at −20 °C until 1- or 2D-GE.

2.3. Isoelectric focusing (IEF)

125 μL rehydration solution (8 M urea, 2% (m/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (Chaps), 2 mM TCEP in deionized water) containing 170 μg protein and 0.1% (v/v) ampholyte (Bio-Lyte 3/10 ampholyte, 100×, Bio-Rad Laboratories) were loaded onto a 7 cm long IPG (immobilized pH gradient) strip, pH range 3–10 (Bio-Rad). After 16 h rehydration the IEF program was started on the Protean IEF cell (Bio-Rad) with a linear voltage ramp to reach 250 V in 20 min. Then, the focusing voltage was further linearly increased to a desired value of 4000 V in 2 h and hold at the maximally attainable voltage for 10,000 Vh. After completion of IEF, the strips were transferred into 2.5 mL of equilibration solution 1 containing 6 M urea, 375 mM tris adjusted to pH 8.8 with 1 M HCl, 2% (m/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 2% (m/v) dithiothreitol (DTT), and gently shaken for 10 min. Equilibration buffer 1 was displaced by equilibration buffer 2 in which the DTT was substituted by 2% (m/v) iodoacetamide. After 10 min shaking, the IPG strip was rinsed with running buffer (composition in Section 2.4), transferred on the top of a 1 mm thick and 7 cm × 10 cm sized polyacrylamide gel, and covered by 0.5% (m/v) molten agarose. The 2nd dimension was proceeded as described in Section 2.4. All chemicals used for gel electrophoresis were purchased from Bio-Rad or Sigma–Aldrich in electrophoresis or molecular biology grade quality.

2.4. SDS-PAGE

For one-dimensional protein separations, samples containing 2.5–100 μg total protein in Lämmli buffer (62.5 mM tris adjusted to pH 6.8 with HCl, 2% (m/v) SDS, 5% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 0.1% (m/v) bromophenol blue) were loaded on 0.75 mm thick polyacrylamide gels that are divided in a 1 or 2 cm long stacking gel (5%, m/v total acrylamide) at the top and a 6 or 18 cm long separation gel at the bottom (15%, m/v total acrylamide) for small or large gel dimensions, respectively. The SDS-PAGE was carried out in the Mini Protean 3 cell (Bio-Rad) for small gels and in the Protean II XL electrophoresis cell (Bio-Rad) for large gels with 25 mM tris adjusted to pH 8.3 with 0.2 M HCl, 192 mM glycine, 0.1% (m/v) SDS as running buffer system at a constant voltage of 150 V for small gels and a current of 18 mA for large gels provided by the Power Pac™ Basic power supply (Bio-Rad). The gels were stained with 0.1% (m/v) Coomassie Brilliant Blue G 250, 0.77 M $(NH_4)_2SO_4$,

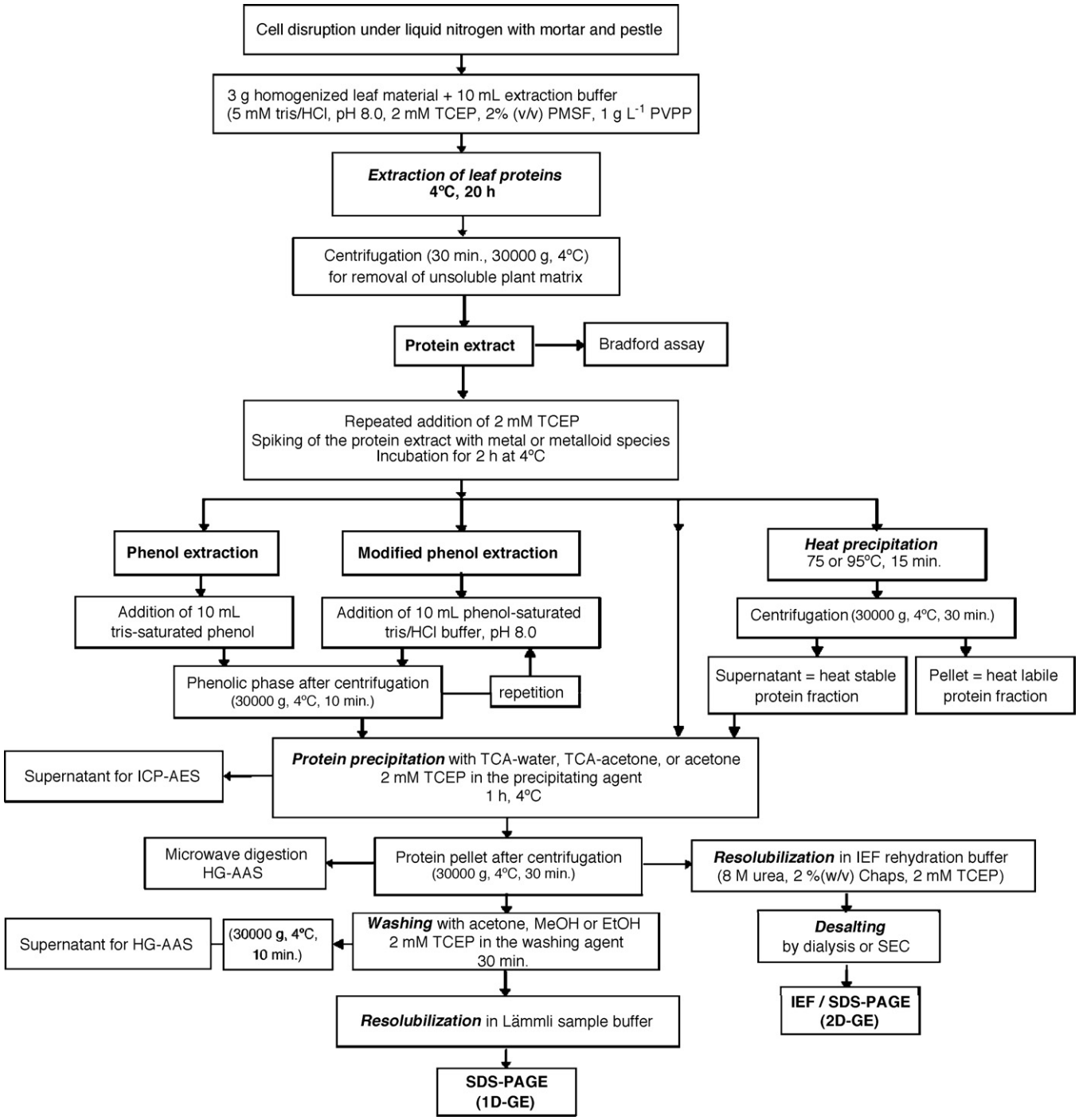

Fig. 1. Flow chart for protein extraction and purification from plant cells for 1D- and 2D-GE analysis.

2% (v/v) H_3PO_4 in 25% (v/v) methanol/75% (v/v) H_2O by shaking over night and destained with deionized water. A molar mass standard comprising seven proteins in the mass range M_r 14–116 (unstained protein relative molecular mass marker, Fermentas) was co-separated with the samples.

2.5. Determination of arsenic and zinc concentrations by atomic spectroscopy

The total element concentrations (As, Zn) were measured during the various steps of the protein preparation procedure (Fig. 1) by inductively coupled plasma atomic emission spectroscopy (ICP-AES, iCAP 6000 series atomic emission spectrometer, Thermo Electron, Dreieich, Germany, with concentric nebulizer and glass cyclone spray chamber) or with atomic absorption spectroscopy with electrothermal atomization (ETA-AAS, ZL 1400, PerkinElmer) for samples with high content of organic solvent and low element concentrations. The AES measurements were conducted at axial plasma, a power of 1350 W, and a sample flow rate of 2 mL min^{-1}, at the wavelengths 189.0, 193.8, and 228.8 nm for As, and 206.2 nm for Zn. The detection limits were 0.7 $\mu g\,L^{-1}$ for As and 0.07 $\mu g\,L^{-1}$ for Zn. For As determination by AAS, the hydride generation technique was used to transfer the analytes in the graphite furnace. The samples were pre-reduced with 5% (m/v) ascorbic acid and 5% (m/v)

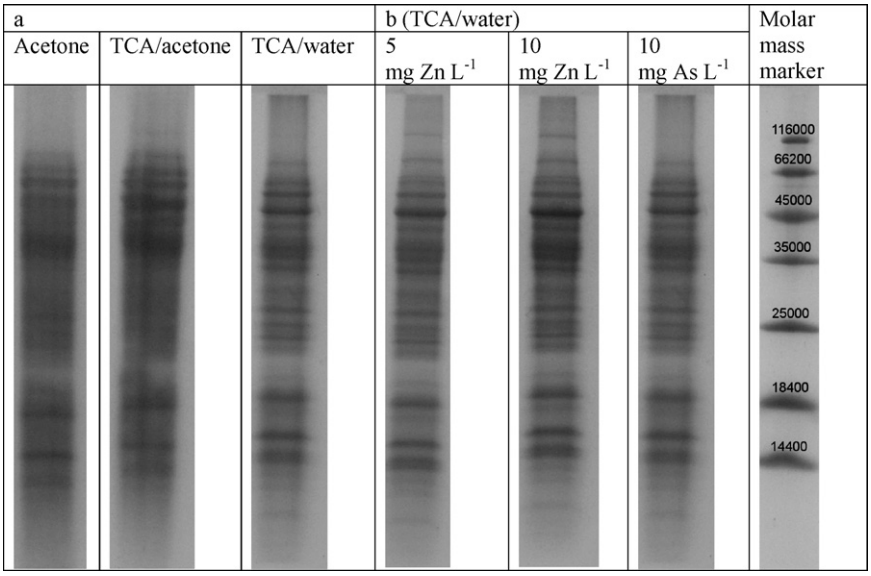

Fig. 2. Influence of the precipitating agent (a), and of the As and Zn addition to the raw protein extract (b) on the SDS-PAGE of *T. majus* leaf proteins. Protein extraction from 3 g fresh leaf tissue with 10 mL 5 mM tris (adjusted to pH 8.0 with 0.2 M HCl), 2 mM TCEP, 2 mM PMSF, 1 g L^{-1} PVPP. 10 μg total protein in 10 μL Lämmli buffer were separated on a 7 cm × 10 cm gel.

potassium iodide for 1 h and then treated with 0.2% (m/v) $NaBH_4$/4% (m/v) HCl. As and Zn were measured at the wavelengths 193.7 nm and 206.2 nm, respectively.

Prior to element quantifications, pellets of protein precipitation were dried up to mass constancy at 50 °C and digested in a microwave device (Microwave system Start 1500, MLS, Leutkirch, Germany) using HNO_3 and H_2O_2 as described previously in Ref. [19].

3. Results and discussion

3.1. Optimization of the protein preparation procedure for 1D- and 2D-GE

The sample preparation procedure was optimized regarding the quality of the 1D- and 2D-GE maps, and a minimization of the effort of time and labor. Unfortunately, except for our previous study [19], no 1D- or 2D-GE results for *T. majus* could be found in the literature for a comparative assessment of our new procedure. The high content of glucosinolates [22] becomes already apparent by the odor of the homogenized plants and is assumed to cause interfering problems with protein electrophoresis.

3.1.1. Variation of the precipitating agent

The protein precipitation by TCA after extraction with aqueous buffer systems is often recommended for plant samples [23,24]. The precipitation step is necessary because many non-protein constituents are present in the raw leaf extract. If the protein precipitation is omitted, no separation is achieved in the 1D-GE [19]. The efficacy of 20% (m/v) TCA in water, 20% (m/v) TCA in acetone, and pure acetone for enrichment and purification of proteins extracted from leaves by the tris buffer system was compared by means of the 1D-GE results (Fig. 2). The protein precipitation with TCA in water delivered the best resolution of protein bands combined with the lowest gel background coloration. Acetonic precipitations also led to a reduced spot number and intensity in 2D gels. After TCA/water and TCA/acetone treatment, the major part of arsenic and zinc spiked to the raw protein extract (81–99% depending on arsenic species and zinc salt) was found in the supernatant of protein precipitation, whereas only 50% of the As concentration of the TCA-containing precipitating agents were detected in pure acetone. Similarly, both TCA precipitations (aqueous and acetonic) resulted in same final As contents of the protein fraction, whereas its binding capacity was 50-fold enhanced by precipitation with TCA-free acetone. These higher As contents of the protein fraction cannot be assigned to an improved protein precipitation efficiency of acetone because the pellet masses were not affected by the type of the precipitating agent. From this it can be assumed that the strong acid diminished the extent of arsenic binding to the protein pellet.

Number and position of the protein bands are not changed by arsenic or zinc spiking of the original protein extract before precipitation (Fig. 2). The 1D-gel electrophoretic separation capacity is not sufficient to resolve metal(oid)-bound proteins from their unbound forms since the changes of molar masses induced by possible metal or metaloid bindings are potentially too small.

3.1.2. Washing of the protein precipitate and desalting of the resolubilized protein

In order to remove co-precipitated compounds, particularly TCA salt from the precipitation step, the pellet was washed with different organic solvents. These washing agents, methanol, ethanol, and acetone, should not dissolve proteins because of their precipitating capacity. However, the kind of the washing agent influenced the residual mass of the precipitate produced by TCA treatment of the protein extracts. The largest pellet mass remained after performing three washing steps with acetone (74 ± 13%, n = 5, of the pellet mass obtained without washing). An enlarged protein loss caused by washing with ethanol and methanol can be assumed since the pellet masses are substantially reduced to 50 ± 3% and 58 ± 9% (n = 6) of the original mass, respectively. Based on these results, acetone was chosen as washing agent for the further experiments. During the washing steps, a small part of arsenic and zinc was resolved from the protein pellet. Thereby, the element concentrations in the wash solutions declined with the number of washing steps. Further, they were affected by the kind of solvent. In the first step, 0.11 ± 0.06% (n = 21) of the arsenic content present in the original protein extract for MMA(V) spiking and 0.34 ± 0.06% (n = 10) of the zinc spiked in the form of zinc acetate were found in the acetonic washing supernatants. During the 2nd and 3rd washing step, the highest As amounts from MMA(V) spiking were dissolved from the protein pellet by ethanol (0.09 ± 0.03%, n = 3, in the 2nd step, and 0.01 ± 0.001%, n = 3, in the 3rd step) followed by acetone (0.04%, in the 2nd step, 0.005% in the 3rd step) and methanol

(0.004 ± 0.0004%, $n = 3$, in the 2nd step, 0.0008 ± 0.0007%, $n = 3$, in the 3rd step).

Good 1D-GE results were achieved after the washing procedure but the washing steps were not sufficient to remove salts and other interfering substances because a high current emerged during IEF and the focusing voltage could not be achieved. For that reason, a desalting step was introduced in the sample preparation protocol. Both desalting variants, small size exclusion chromatography columns or dialysis throughout a 1 kDa-cut-off membrane, significantly reduced the IEF current. The desalting with the PD-10 columns led to poorly reproducible 2D-GE patterns because the ratio of the protein spots to the background gel staining fluctuated between different samples. The desalting by dialysis brought the crucial break-through to get reproducible, well resolved 2D-GE results. If the washing steps were omitted before dialysis the focusing voltage was achieved but some horizontal and vertical streaking arrived in the acidic region of the 2D map. The bromophenol blue added to the samples before IEF changed to a green color probably due to TCA residues. A short manual shaking of the protein pellet in 2 mL acetone sufficed to prevent the discoloration of bromophenol blue and to produce the sharpest contrast between protein spots and gel background (Fig. 3). Similar as in case of the 1D-GE experiments (see Fig. 2), the arsenic addition had no remarkable effect on that protein pattern.

3.1.3. Comparison of tris–TCA extraction, phenol-assisted extraction variants, and heat precipitation

The phenol extraction method proved to be a good choice for proteomic studies of fruit and leaf tissues characterized by a high content of interfering metabolites [15,21,23]. Two phenol-assisted extraction variants were tested regarding a possible improvement of the 2D-GE results of *T. majus* proteins (Fig. 1). The conventional phenol extraction [21] did not deliver good results since the TCA precipitation of the phenolic phase produced a very small protein yield. On the other hand, the protein extraction with the phenol-saturated tris buffer produced highly resolved 2D-GE maps with low background staining but the spot number was reduced compared to simple TCA precipitation. The creation of a protein-enriched phenolic phase (Fig. 1) resulted in an enhanced arsenic concentration of the final protein pellet (Fig. 4). But due to the toxicity of the phenol and the enhanced labor effort, the phenol-assisted extraction cannot be recommended for the 2D-GE investigation of *T. majus* proteins. The heat precipitation revealed that both generated protein fractions possess a strongly enhanced arsenic-binding capacity. Unfortunately, no evaluable 1D- and 2D-GE results could be obtained up to now from the heat-stable protein fraction due to the very small protein yield.

Summarizing, the following parameters were chosen for the optimized sample preparation protocol for 1D- and 2D-GE—extraction buffer system: 5 mM tris adjusted to pH 8.0 with 0.2 M HCl, 2 mM TCEP, 2 mM PMSF, 1 g L^{-1} PVPP; protein precipitation: 20% (m/v) TCA in water; washing of the protein precipitate: 2 mL acetone; desalting: dialysis. This procedure delivered not the highest As contents in the protein fraction but the best resolution and reproducibility of the 2D-GE results. The optimized protein preparation method also delivered satisfying gel electrophoretic results for other plant sample types (Fig. 5). The intensive band below the molar mass marker at M_r 18,400 in the 1D-gels could be assigned to sunflower albumin (M_r between 10,000 and 18,000) which accounts for 10–30% of the total protein content of sunflower seeds [25]. Similar as for *T. majus* leaves, the desalting of the protein samples from sunflower seeds by dialysis led to a higher quality of the 2D-GE maps compared to desalting by small SEC columns (Fig. 6). The intensive smearing of the PD-10 desalted samples could be diminished by up to 10-fold dilution with rehydration solution but thereby many pro-

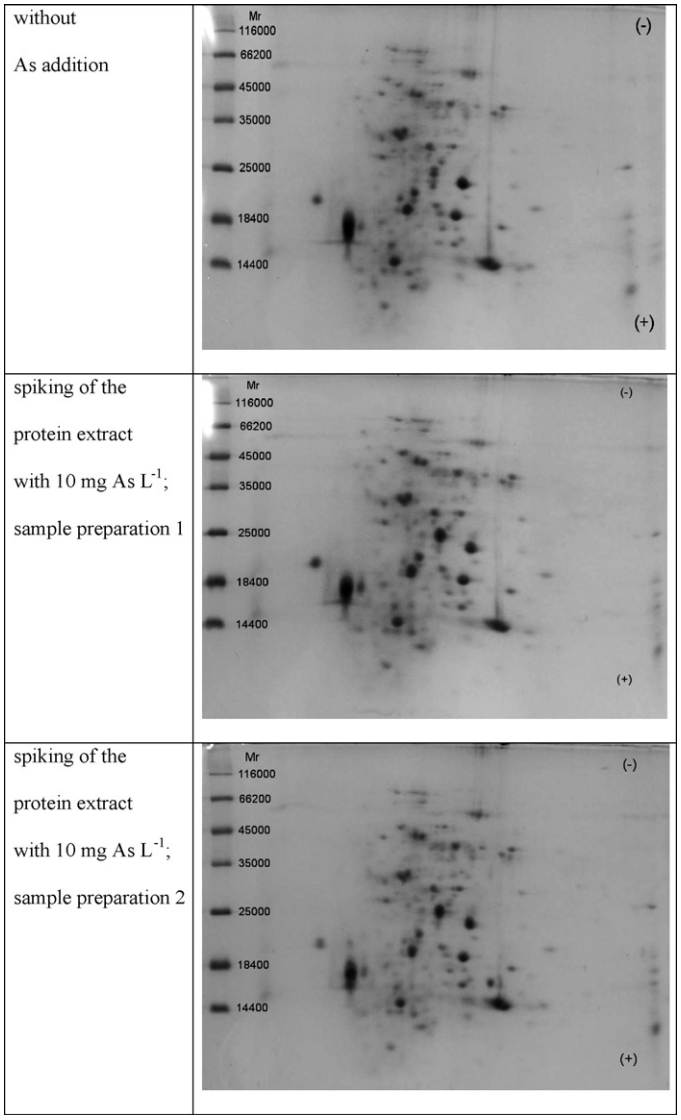

Fig. 3. 2D-GE maps of leaf proteins extracted from *T. majus* by 5 mM tris (adjusted to pH 8.0 with 0.2 M HCl), 2 mM TCEP, 2 mM PMSF, 1 g L^{-1} PVPP. Protein precipitation with TCA in water; one washing step with acetone, dialysis.

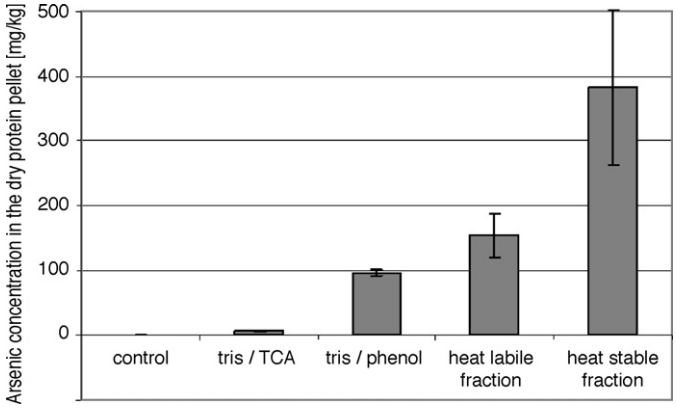

Fig. 4. Arsenic concentrations in the dried protein pellets of *T. majus* leaves resulting from different precipitation methods after protein extraction with 5 mM tris (adjusted to pH 8.0 with 0.2 M HCl), 2 mM TCEP, 2 mM PMSF, 1 g L^{-1} PVPP and arsenic spiking of the raw protein extract (10 mg As L^{-1} as MMA(V)). Average values with standard deviations from 3 to 8 parallel experiments are shown. *Control*: No As spiking; TCA/water precipitation. TCA: TCA/water precipitation. Phenol/TCA: phenol extraction and TCA/water precipitation. Heat labile fraction: heat precipitation. Heat stable fraction: TCA/water precipitation after heat precipitation.

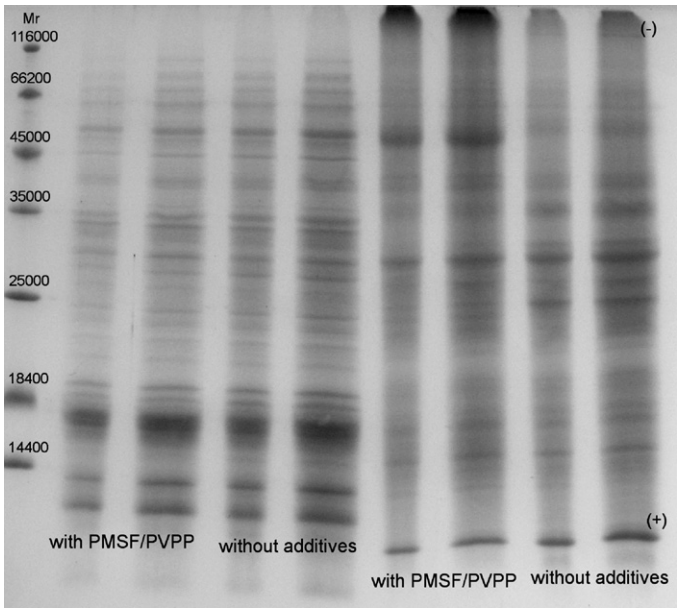

Fig. 5. SDS-PAGE of proteins extracted from sunflower seeds and from red fescue by 5 mM tris (adjusted to pH 8.0 with 0.2 M HCl), 2 mM TCEP, with or without 2 mM PMSF and 1 g L^{-1} PVPP. Protein precipitation by TCA in water. 50 and 100 μg total protein for each sample were separated on a 20 cm × 20 cm gel.

tein spots disappeared. Also here, the protein pattern in the 2D gel did not change through As or Zn spiking of the raw protein extracts.

3.2. Influence of different arsenic species and zinc salts on the As and Zn incorporation in the protein fraction

The As and Zn contents of the final protein fractions and the corresponding wash solutions are compared for spiking of raw protein extracts with different arsenic species and zinc salts and for arsenic species application during plant growth in Fig. 7. In the spiking experiments, the trivalent arsenic compounds As(III) and PAO were bound to the proteins to a considerably larger extent than the pentavalent species As(V), MMA(V), and DMA(V). The AAS analyses revealed a slightly stronger binding of Zn to the protein pellets compared to pentavalent As. Within the different zinc salts, the extent of Zn binding to the protein pool is inversely correlated with the strength of the corresponding acids in the order chloride > sulfate > acetate due to a suppression of Zn^{2+} complexation by protein protonation. The dimethylated arsenic compound caused the lowest arsenic-binding capacity of the protein pool both in case of spiking and application for both plant sample types. In general, a much higher part of the arsenic present in the original protein extract was recovered in the protein fraction in case of arsenic application during plant growth compared to arsenic addition to the protein extracts. The total extractable leaf protein concentrations of plants grown under exposure to different arsenic species and of unpolluted control plants did not differ. Moreover, a lower part of arsenic was resolved in the washing step if the plants accumulated the arsenic during growth indicating a stronger binding to proteins than in case of arsenic addition to the protein extract.

The element contents originally present in the raw protein extracts agreed with the sum of contents in the produced fractions (Table 1). Because the element concentrations measured in the raw extracts and in the supernatants of precipitation in the mg L^{-1} range were one order of magnitude larger than the μg L^{-1} concentrations in the wash solutions and in the protein pellets, the AAS and AES analyses delivered reliable results.

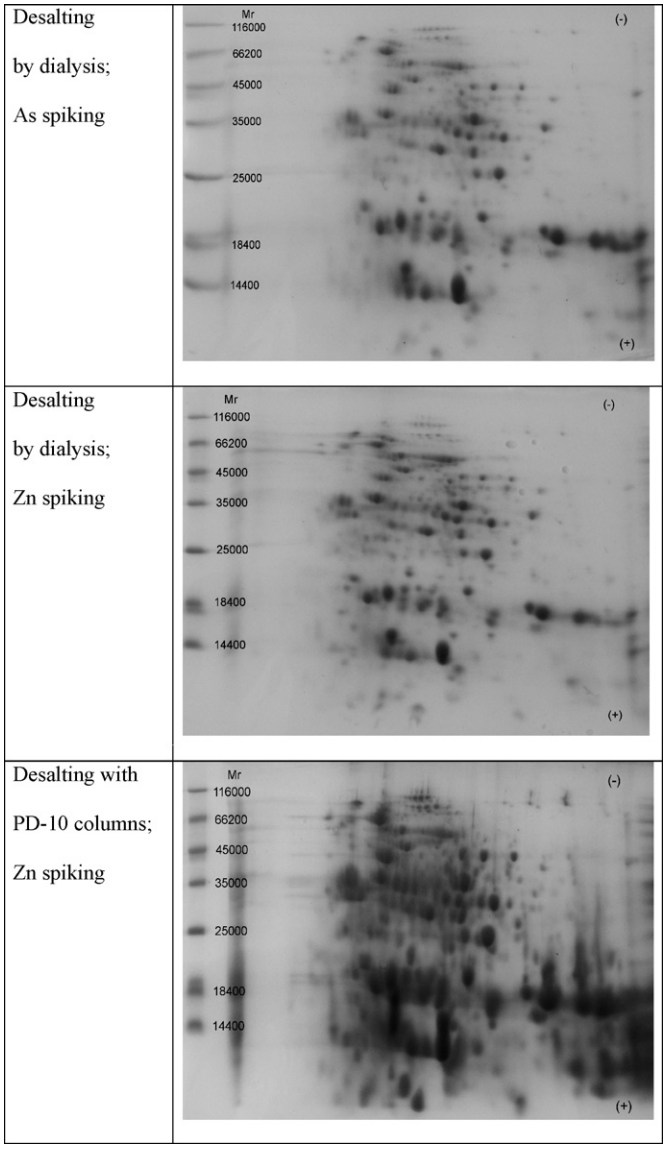

Fig. 6. Effects of the desalting variant on the 2D-GE maps of sunflower seed proteins spiked with As or Zn. Protein extraction with 5 mM tris (adjusted to pH 8.0 with 0.2 M HCl), 2 mM TCEP, 2 mM PMSF, 1 g L^{-1} PVPP. Protein precipitation with TCA–water; three washing steps with acetone.

Despite of the arsenic incorporations into the protein pool demonstrated by microwave-digestion and AAS analysis, no changes in the leaf protein patterns of plants cultivated under exposure to different arsenic species could be ascertained by the current procedure of 1D- and 2D-GE.

Table 1
Balances of As and Zn contents determined by AAS and AES in the original protein extract and in the different fractions of the protein preparation procedure for selected samples.

Sample	As or Zn content [μg]	
	Raw protein extract	Sum (supernatant of precipitation + wash solution + protein fraction)
MMA(V) application, *T. majus* leaves	20.1 ± 1.7	20.0
DMA(V) application, *T. majus* leaves	18.1 ± 2.8	18.9
As(V) application, *T. majus* leaves	4.4 ± 0.8	5.5
As(III) application I, *T. majus* leaves	9.6 ± 0.9	10.6
$Zn(CH_3COO)_2$ spiking, *T. majus* leaves	97.8 ± 10.4	99.8
$Zn(CH_3COO)_2$ spiking, *H. annuus* seeds	113.1 ± 28.1	114

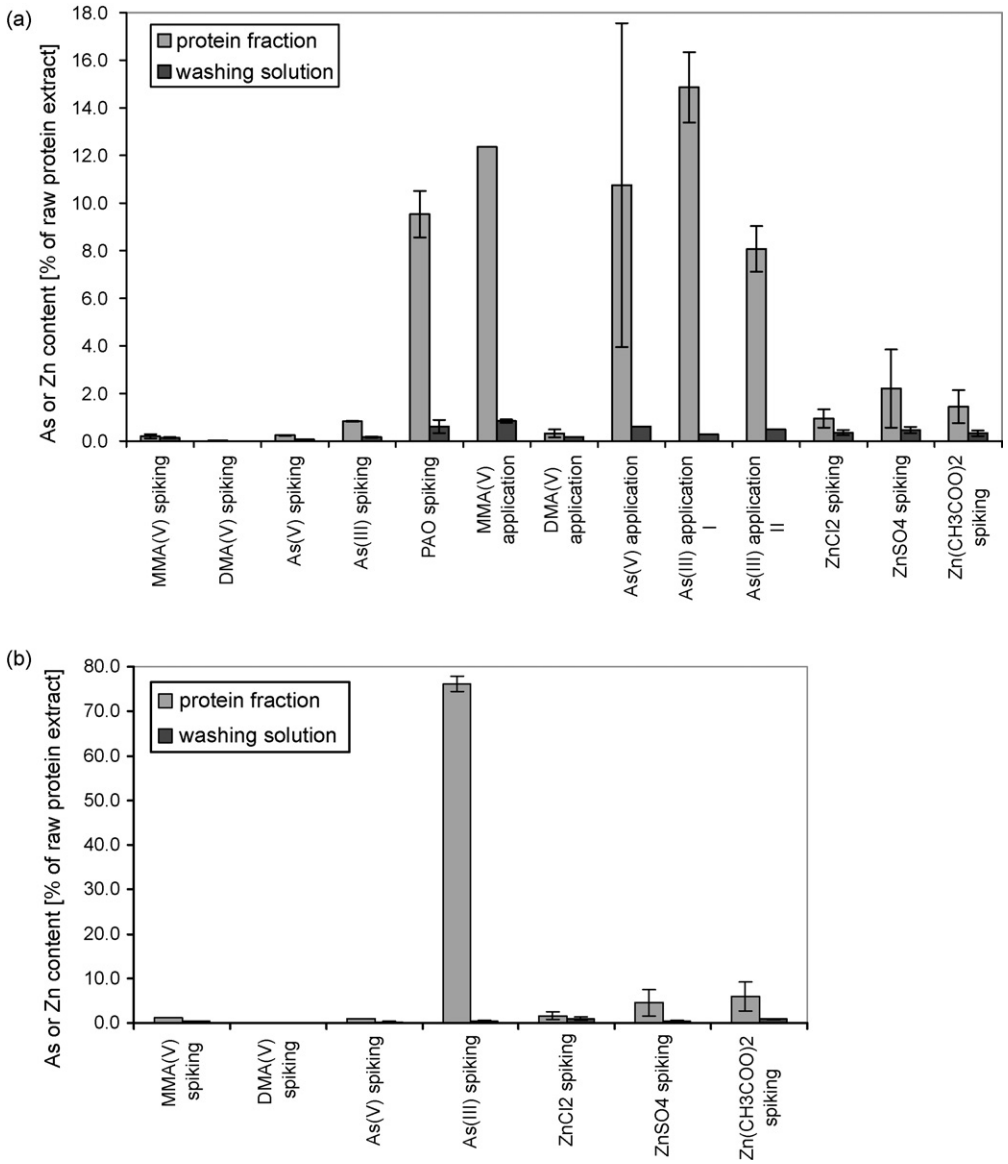

Fig. 7. Relative arsenic and zinc contents in the final protein fractions produced by tris buffer extraction and TCA/water precipitation for *T. majus* leaves (a) and *H. annuus* seeds (b) after spiking of the raw protein extract with arsenic species or zinc salts and after application of different arsenic species during plant growth. As and Zn contents in the original protein extracts were 100 μg in 10 mL for spiking experiments, 20.1 ± 1.7 μg in 10 mL (n = 3) for MMA(V) application, 18.1 ± 2.8 μg in 10 mL (n = 3) for DMA(V) application, 4.4 μg in 10 mL for As(V) application, 9.6 ± 0.9 μg in 10 mL (n = 6) for As(III) application I, and 9.4 ± 1.1 μg in 10 mL (n = 4) for As(III) application II. Protein concentrations: 3297 ± 268 μg mL^{-1}, n = 8, for *H. annuus* seed extracts; 907 ± 107 μg mL^{-1}, n = 16, for *T. majus* leaf extracts.

3.3. Concluding remarks

A sample preparation scheme including protein extraction, precipitation, washing, and desalting was developed that provides well-resolved and reproducible 1D- and 2D-GE maps for different kinds of plant matrices such as nasturtium leaves, grass, and sunflower seeds. The arsenic and zinc distribution over the different steps of this protein purification procedure could be determined. The kind of protein precipitation affected the extent of arsenic binding to the produced protein fraction. The TCA/water precipitation that delivered the best 2D-GE results led to the lowest arsenic contents of the protein precipitate. Therefore, more investigations are necessary to characterize the nature of the bindings. Plants cultivated under the exposure to different arsenic species are characterized by stronger arsenic bindings to the protein pool in comparison to arsenic spiking of the protein extracts. In addition, the kind of arsenic species influenced the binding capacity of the proteins. In the next step, more sensitive protein stainings are necessary to visualize possible arsenic- and zinc-influenced proteins in 2D gels. In the future work, the gels should also be scanned for elevated arsenic and zinc concentrations by LA–ICPMS.

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