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Influence of one- and two-dimensional gel electrophoresis procedure on metal–protein bindings examined by electrospray ionization mass spectrometry, inductively coupled plasma mass spectrometry, and ultrafiltration

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ABSTRACT

Three independent methods, (i) electrospray ionization mass spectrometry (ESI-MS), (ii) carrying out the complete protein preparation procedure required for protein gel electrophoresis (GE) including extraction, precipitation, washing, and desalting with subsequent microwave digestion of the produced protein fractions for metal content quantification, and (iii) ultrafiltration for separating protein-bound and unbound metal fractions, were employed to elucidate the influences of protein sample preparation and GE running conditions on metal–protein bindings. A treatment of the protein solution with acetone instead of trichloroacetic acid or ammonium sulfate for precipitate formation led to a strongly enhanced metal binding capacity. The desalting step of the resolubilized protein sample caused a metal loss between 10 and 35%. The omission of some extraction buffer additives led to a diminished metal binding capacity of protein fractions obtained from the sample preparation procedure for GE, whereas a tenside addition to the protein solution inhibited metal–protein bindings. The binding stoichiometry of Cu and Zn–protein complexes determined by ESI-MS was influenced by the type of the metal salt which was applied to the protein solution. A higher pH value of the sample solution promoted the metal ion complexation by the proteins. Ultrafiltration experiments revealed a higher Cu- and Zn-binding capacity of the model protein lysozyme in both resolubilization buffers for 1D- and 2D-GE compared to the protein extraction buffer. Strongly diminished metal binding capacities of lysozyme were recorded in the running buffer of 1D-GE and in the gel staining solutions.

1. Introduction

What function a biomolecule fulfills, often depends on its specific, non-covalent interaction with other molecules such as peptides and other proteins, polysaccharides and lipids, or with metal ions [1]. Metal binding proteins are responsible for many processes in metabolism, such as energy transfer during photosynthesis and respiration or signal transfer processes which control gen expression and regulation. In proteins, metal ions can act as catalysts or regulators for the transport and storage of substances [2]. Metal bindings control the biological functionality of proteins by blocking or activation of catalytical centres or by changing the sterical folding structure. Besides essential metal functions, also toxic effects can occur, e.g. in plants growing on heavy metal-contaminated areas. The interactions between proteins and metal ions can be examined with one- or two-dimensional gel electrophoresis (1D- or 2D-GE) with subsequent laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). After gel electrophoretic separation, protein bound metal ions can be identified in the separated protein spots by LA-ICP-MS [3,4]. The two-dimensional gel electrophoresis (2D-GE) offers a high resolution for separating both low- and high-molecular mass proteins what enables the analysis of the whole proteom of cells or tissue types. But the crucial step to obtain reproducible and well resolved GE results exists in the sample preparation. The proteins have to be extracted from the biological material and then they must be precipitated. In some procedures, the precipitation step is omitted [5] but often this step is required to purify and to concentrate the proteins [6]. The obtained protein pellet is washed and dissolved in GE resolubilization buffer [6]. However, during this protein preparation procedure from biological materials several chemicals are needed which can change the original binding state of metal–protein complexes. Also during GE, metal–protein bindings can dissociate since components of the buffer solutions can coordinate with the metal ions. First studies directed towards the evaluation of stabilities of metal–protein bindings under condi-

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tions of gel electrophoresis point out dissociating effects occurring during sample preparation [7], 1D-GE runs [5,8], as well as gel staining [9]. So the question remains to what extent the results obtained by GE-LA-ICP-MS concerning the binding behavior of metal ions to proteins reflect the interaction of proteins and metal ions in their natural environment. Our former investigations concerning the influencing variables of the sample preparation on the arsenic and zinc binding capacity of a complex plant protein mixture revealed a strong influence of the kind of protein precipitation [6]. In the current study, influences of protein extraction, precipitation, and desalting of the resolubilized protein as well as of buffer systems used in 1D- and 2D-GE on interactions of proteins and heavy metal ions (Cu, Zn) are examined by three independent methods. In order to afford a more clearly effect interpretation compared to complicated real plant samples, the system was simplified to some model proteins characterized by different molar masses and p*I* values. The methods used for investigation of metal–protein interactions comprised (i) electrospray ionization mass spectrometry (ESI-MS) of metal containing protein solutions, (ii) metal content quantification in protein fractions resulting from the protein preparation process for 1D and 2D-GE, and (iii) ultrafiltration for separating unbound and protein-bound metal fractions in different GE buffer systems. Because of the gentle ionization process, also weak protein–metal complexes can be analyzed by ESI-MS. The molecular mass of the complex can be determined and the stoichiometry of the complex building components can be derived. Changes of protein conformation can also be recognized by ESI-MS [1]. A disadvantage especially for investigation of the influences of components needed for 1D and 2D-GE on protein–metal bindings is its incompatibility for non-volatile salts, denaturants such as urea [10] and detergents [11]. By using ESI-MS, the effects of the anion of the applied metal salts as well as of the redox and pH conditions on protein–metal interactions were elucidated in the current study. A second possibility for the investigation of the influence of the composition of the extraction buffer and the precipitating agent on protein–metal interactions is to carry out the different steps of protein extraction and precipitation with the aim to determine the metal concentration in produced protein pellets after microwave digestion [6,12]. By this way, the binding capacity of the protein for the metal ions can be ascertained. The influence of the various buffer systems to which the metal-binding proteins are exposed during 1D- and 2D-GE separations including the staining procedure can be investigated by ultrafiltration over size exclusion membranes. The unbound metal fraction which is collected in the filtrate can be quantified by ICP-MS. With the ultrafiltration method, also influences of such components can be recorded that show an incompatibility for ESI-MS measurements.

2. Materials and methods

2.1. ESI-MS

The time-of-flight (TOF) mass spectrometer (Mariner™, Applied Biosystems, Houston, TX, USA) was tuned and mass-calibrated daily to optimize signal intensity and resolution using a peptide mixture made of angiotensin, neurotensin and bradykinin of 1 μM each. Mass spectrometric metal binding studies were performed with hen egg white lysozyme (Lys), bovine α-lactalbumin (α-Ltb), horse heart cytochrome c (Cyt c) and bovine serum albumin (BSA) in a concentration of 10 μM in acetonitrile (ACN, HPLC gradient grade, VWR International)/deionized water/acetic acid (HAc, p.a., Merck, Darmstadt, Germany) (50:49:1, v/v/v). All proteins as well as acetate salts of the tuning peptides were purchased from Sigma–Aldrich (Steinheim, Germany) as lyophilized powders and stored at −18 °C as aqueous 100 μM stock solutions. The heavy metals Cu(II) and Zn(II) were added to the 10 μM protein sample solutions to final concentrations between 200 and 750 μM. For this purpose, 1–10 mM metal stock solutions were prepared from zinc chloride (≥98.0%, Sigma–Aldrich), zinc acetate dihydrate (≥99.5%, Sigma–Aldrich), zinc sulfate heptahydrate (≥99.0%, Sigma–Aldrich), zinc nitrate hexahydrate (98%, Sigma–Aldrich), copper(II) chloride dihydrate (≥99.0%, Sigma–Aldrich), copper(II) acetate monohydrate (≥99%, Sigma–Aldrich), copper(II) sulfate pentahydrate (≥99.0%, Sigma–Aldrich) and copper(II) nitrate trihydrate (p.a. quality, Merck) and stored at 4 °C.

All measurements were carried out at a nozzle temperature of 140 °C, a nozzle potential of 100 or 120 V and a spray tip potential of 4000 V. The flow rate of the syringe pump injection was either 5 or 10 μL min^{-1}. For each sample 10 mass spectra were acquired at an acquisition time of 3 s per spectrum. The supplying capillary and the ESI needle were flushed with a solution of 49% H_2O, 50% ACN and 1% Hac (v/v/v).

For the investigation of an influence of the oxidation state of the cysteine residues of the proteins on their metal interactions, the protein solutions in 49% H_2O/50% ACN/1% Hac (v/v/v) were incubated with the reducing agent TCEP (tris(2-carboxyethyl)phosphine hydrochloride solution, 0.5 M, Sigma–Aldrich) in different concentrations between 8 and 40 μM for 0.5 h before addition of the metal solution.

The pH value of the metal-containing protein samples was varied from 4 to 7 by adjusting the pH of a 5 mM ammonium acetate buffer before ACN addition in a volume ratio of 1:1.

2.2. ICP-MS

ICP-MS (inductively coupled plasma mass spectrometry) measurements were performed with the ICP mass spectrometer X Series 2 (Thermo Fisher Scientific) equipped with an autosampler (ASX-520, Cetac), a concentric standard glass nebulizer and a collision cell. The mass spectrometer was tuned with an element standard solution containing 10 μg L^{-1} of In, U, Ba, and Ce, each, to reach signal intensities >400 000 cts for In (*m/z* 115) and >800 000 cts for U (*m/z* 238), a CeO rate of <2% and a Ba^{2+} rate < 3%. An external calibration for the analytes Zn and Cu was carried out with standard solutions containing 1, 10, 50, 100 and 500 μg L^{-1} of each element. The signal intensities of ^{66}Zn and ^{63}Cu were measured in the KED (kinetic energy discrimination) mode using an energy barrier of 2 V between the hexapole bias and the pole bias and related to the intensity of the internal standard ^{74}Ge (50 μg L^{-1}) to correlate the instrumental drift over the day and possible sample matrix effects on the nebulization. After each sample measurement, the feeding PEEK capillary and the nebulizer were flushed with 2 vol.% HNO_3. The instrumental parameters are specified in Table 1.

2.3. Protein preparation procedure: extraction, precipitation, washing, and desalting

To investigate the influence of the different steps of the protein preparation for gel electrophoresis on protein metal interactions, Lys ($M \approx 14.3$ kDa [13]; pI = 10.7 [14]) and BSA ($M \approx 67$ kDa [15]; p$I \approx 4.5$–4.9 [16]) were used as model proteins. The sample preparation was carried out in 30 mL polypropylene centrifuge tubes

Table 1
Instrumental parameters of the ICP-MS for Cu and Zn measurements in the KED 2 V mode.

Collision gas	7% H_2 in He (purity 5.0)
Collision gas flow	5 mL min^{-1}
Nebulizer flow	1 L min^{-1} Ar
Forward power	1400 V
Dwell time	20 ms
Measurements per sample	3

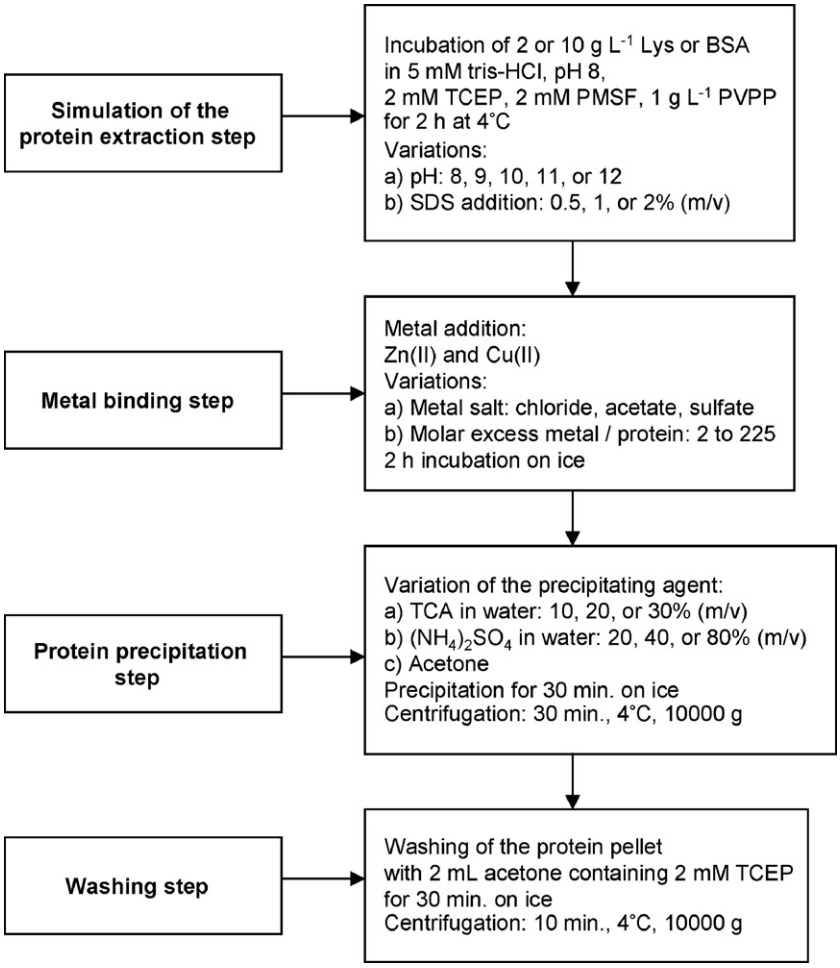


Fig. 1. Protein preparation procedure for determination of the metal binding capacity of model proteins under the conditions of 1D- and 2D-GE sample preparation.

(Nalge Nunc International Corporation, Rochester, NY, USA). During all steps of the protein preparation procedure that are described in Fig. 1, a concentration of the reducing agent TCEP of 2 mM was maintained. The composition of the extraction buffer as well as the washing and resolubilization procedure was adopted from our previous work [6,12]. The tris (tris(hydroxymethyl)aminomethane, p.a. quality from Merck) buffer was adjusted to pH 8.0 using 1 M hydrochloric acid (diluted from 32% m/v HCl, p.a. quality from Sigma–Aldrich). The extraction buffer additives PMSF (phenylmethylsulfonyl fluoride, 0.1 M stock solution in ethanol), PVPP (polyvinylpolypyrrolidone), and sodium dodecyl sulfate (SDS; ≥98%) were purchased from Sigma–Aldrich. The precipitating agents TCA (trichloroacetic acid, ≥99.5%, from Sigma–Aldrich) and ammonium sulfate (p.a., from Merck) were added to the protein sample as differently concentrated aqueous solutions in a volume ratio of 1:1, whereas a 4-fold volume excess of pure acetone (p.a., from Merck) was provided to the sample solution. A Heraeus Biofuge Primo R centrifuge (Thermo Scientific) was used. Metal stock solutions of 4.5 and 45 g Zn or Cu L^{-1} were prepared using the mentioned chloride, acetate, and sulfate salts (see Section 2.1) and stored at 4 °C.

In the protein preparation procedure for 1D- and 2D-GE, the obtained protein pellet is resolubilized in 1D- or 2D-GE sample buffer for loading onto the sample slots of 1D gels or onto IEF strips. In order to determine the metal contents of the produced protein fractions, the washed pellets were dried in a drying oven at 50 °C until mass constancy. Because the protein pellet was contaminated by unsoluble PVPP originating from the simulated extraction step, the mass of added PVPP was subtracted from the final mass weighted for the dried protein pellet. The dried pellets were digested in a microwave device (Microwave system Start 1500, MLS, Leutkirch, Germany) using HNO_3 (65%, p.a., from Merck) and H_2O_2 (30%, suprapur, from Merck) as described previously [12]. Metal concentrations of the digests were quantified by ICP-MS (Section 2.4) after 1:3 up to 1:1000 dilution with deionized water. To get the blank values for the binding capacity of PVPP for Zn^{2+} and Cu^{2+} ions, the whole protein preparation procedure with subsequent microwave digestion was carried out without protein. The metal concentration of the PVPP precipitate was subtracted from the metal contents measured in mixed protein-PVPP pellets.

In parallel, 10 mg of some protein pellets were resolved in 3700 μL 2D-GE resolubilization buffer consisting of 8 M urea (solid substance in GE quality from Merck), 2% (m/v) CHAPS (3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate, ≥98%, Sigma–Aldrich) and 2 mM TCEP. This protein solution was desalted in dialysis tubes (Mini Dialysis Kit, 1 kDa cut-off, 2 mL, Amersham Biosciences) against 8 M urea. After 4 h stirring at room temperature using a magnetic stirrer, the urea solution was renewed and the samples were further dialyzed at 4 °C overnight. The Zn and Cu concentrations of the samples before and after the dialysis were determined by ICP-MS (Section 2.4).

2.4. Ultrafiltration

To examine the effect of the buffer systems used during gel electrophoresis (Table 2) on protein metal interactions, ultrafiltra-

Table 2
Composition of 1D- and 2D-GE buffer systems examined by ultrafiltration regarding their effect on Cu and Zn bindings to lysozyme.

Buffer system	Composition of the buffer systems	Incubation time
Buffer for protein extraction	5 mM tris adjusted to pH 8.0 using 1 M HCl 2 mM TCEP 2 mM PMSF [6]	2 h
Resolubilization buffer for 1D-GE	62.5 mM tris adjusted to pH 6.8 using 1 M HCl 2% (m/v) SDS 5% (v/v) β-mercaptoethanol[a] 20% (v/v) glycerol[b] 0.1% (m/v) bromophenol blue[c] [6]	2 h
Resolubilization buffer for 2D-GE	8 M urea 2% (m/v) CHAPS 2 mM TCEP [6]	2 h
Running buffer for 1D-GE	25 mM tris adjusted to pH 8.3 using 1 M HCl 192 mM glycine[d] 0.1% (m/v) SDS [6]	30 min
Equilibration buffer 1 between 1st and 2nd dimension	6 M urea 375 mM tris adjusted to pH 8.8 using 1 M HCl 2% (m/v) SDS 20% (v/v) glycerol 2% (m/v) DTT (dithiothreitol)[e] [6]	30 min
Equilibration buffer 2	6 M urea 375 mM tris adjusted to pH 8.8 using 1 M HCl 2% (m/v) SDS 20% (v/v) glycerol 2% (m/v) iodoacetamide[f] [6]	30 min
Coomassie staining	0.1% (m/v) Coomassie® Brilliant Blue G 250[g] 0.77 M ammonium sulfate 2% (v/v) phosphoric acid[h] 25% (v/v) methanol[i] [6]	1 h
Silver staining	0.1% (m/v) silver nitrate 0.01% (v/v) formaldehyde[j] [30]	30 min

Solid substances: [a]≥98%, [b]≥99%, [c]sodium salt, for electrophoresis, [d]≥99%, [e]≥99%, [f]molecular biology grade, [g]≥85%; [a–g]from Sigma–Aldrich; [i]HPLC Gradient grade, Fisher Scientific, [j]35% solution, stabilized with 10% MeOH, from Riedel de Haën.

tion over size exclusion membranes was carried out. 1.4 or 2 g L^{-1} lysozyme were dissolved in each buffer solution and incubated with Zn(II) or Cu(II) chloride in a 20-fold molar excess to the protein according to the time in which the protein is exposed to the buffer systems during GE (Table 2). Then the solution was transferred into Vivaspin concentrator tubes (Vivaspin 6, Vivascience, Sartorius group, Stonehouse, UK; sample volume 2–6 mL, polyethersulfone membrane, 3 kDa cut-off) and centrifuged at 10,000 × *g* and 4 °C for 30–70 min to concentrate the protein onto the membrane and to collect the filtrate in the bottom part of the tubes. The metal concentration of the filtrate was determined by ICP-MS (Section 2.4). Blank values, that means the Cu and Zn contents of the examined buffer systems, were measured and subtracted from the protein–metal solutions.

3. Results and discussion

3.1. ESI-MS

The mass spectrometric measurements demonstrated that Zn^{2+} and Cu^{2+} ions interact with hen egg white lysozyme, bovine α-lactalbumin, horse heart cytochrome c and bovine serum albumin. Because an influence of the type of the salt anion on the zinc binding capacities of plant proteins was ascertained in our former studies [6], the metal salt was varied during the ESI-MS analyses. The obtained mass spectra exhibit an influence of the salt anion on the binding stoichiometry of the metal–protein complexes (Table 3). The largest number of both metal ions was associated to the proteins, if the metals were added to the protein solution in the form of acetate salts. Within the series of the other tested salt types (chlorides, sulfates, and nitrates) no definite trend could be deduced from the mass spectra concerning the binding stoichiometry. Possibly, owing to the lower acid strength of acetic acid compared to the corresponding acids of the other salts, a lower proton activity competes with metal cation complexation at protein binding sites even if the sample buffer concentration exceeded the metal salt concentration at least 6.7-fold. Another reason could be ascribed to the fact that acetate did not form adducts with proteins in contrast to all other used salt anions. If the metal ions were added to the protein solution as chlorides, sulfates, or nitrates, it was observed that both metal ions and the corresponding anions were bound to the proteins. The deconvoluted ESI mass spectrum of Lys under addition of Cu(II) chloride in a 75-fold molar excess of Cu against Lys in Fig. 2a shows the association of up to five chloride ions and four Cu^{2+} ions. If Zn(II) chloride in a 75-fold molar excess of Zn against Lys was added, the binding of three chloride ions and only one Zn^{2+} ion was detected (mass spectrum not shown). Chloride ions can for example bind to the amino acid side chain of lysine [17]. The binding of chloride ions to proteins was also observed for α-Ltb and Cyt c. So the disadvantage of using metal chlorides exists in the formation of protein chloride adducts which complicates the interpretation of the mass spectra. Fig. 2b shows the ESI mass spectrum of Lys under addition of Zn(II) sulfate in a 20-fold molar excess of Zn against Lys. There the bindings of up to five sulfate ions and only one Zn^{2+} ion can be observed. Sulfate ions can bind to proteins by hydrogen bonds between the oxygen atoms of the sulfate ion and the NH groups of the polypeptide chain, the OH group of serine or the NH group of tryptophane [18]. Moreover, the sulfate adduct peaks exhibit a dominant intensity. In case of Cu(II) sulfate addition in a 20-fold molar excess of Cu against Lys, the same number of sulfate (5) and Cu (1) ions interacted with Lys (mass spectrum not shown) as recorded for Zn(II) sulfate above. Due to the high intensity of the peaks of protein sulfate adducts, it was difficult to recognize protein metal adducts. The same problem was observed in the ESI mass spectra of α-Ltb and Cyt c under addition of Zn(II) and Cu(II) sulfate.

Table 3
Maximal number of metal ions bound to Lys, α-Ltb, and Cyt c in dependence on the metal salt type and the metal excess determined by ESI-MS in 49% H_2O/50% ACN/1% HAc (v/v/v), pH 2.8, or in 5 mM 50% NH_4 Ac/50% ACN (v/v), pH 4.0 or 7.0.

Protein	Metal salt	Molar excess metal/protein	pH[a]	Maximal number of bound metal ions to one protein molecule	
				Zn(II)	Cu(II)
Lys	Acetate	20	2.8	2	3
		50	4.0	n.m.	4
		50	7.0	5	5
	Chloride	20	2.8	1	2
		50	4.0	2	3
		50	7.0	3	4
		75	2.8	1	4
	Sulfate	20	2.8	2	1
		50	7.0	2	2
	Nitrate	75	2.8	2	2
α-Ltb	Acetate	50	2.8	3	4
		50	4.0	4	n.m.
	Sulfate	50	2.8	2	2
	Nitrate	50	2.8	2	2
Cyt C	Acetate	50	2.8	3	3
		50	7.0	4	6
	Chloride	10	2.8	1	1
	Sulfate	50	7.0	3	3
	Nitrate	50	2.8	3	3

[a] The pH value of the sample solution was measured before ACN addition in the aqueous phase. n.m.: not measured.

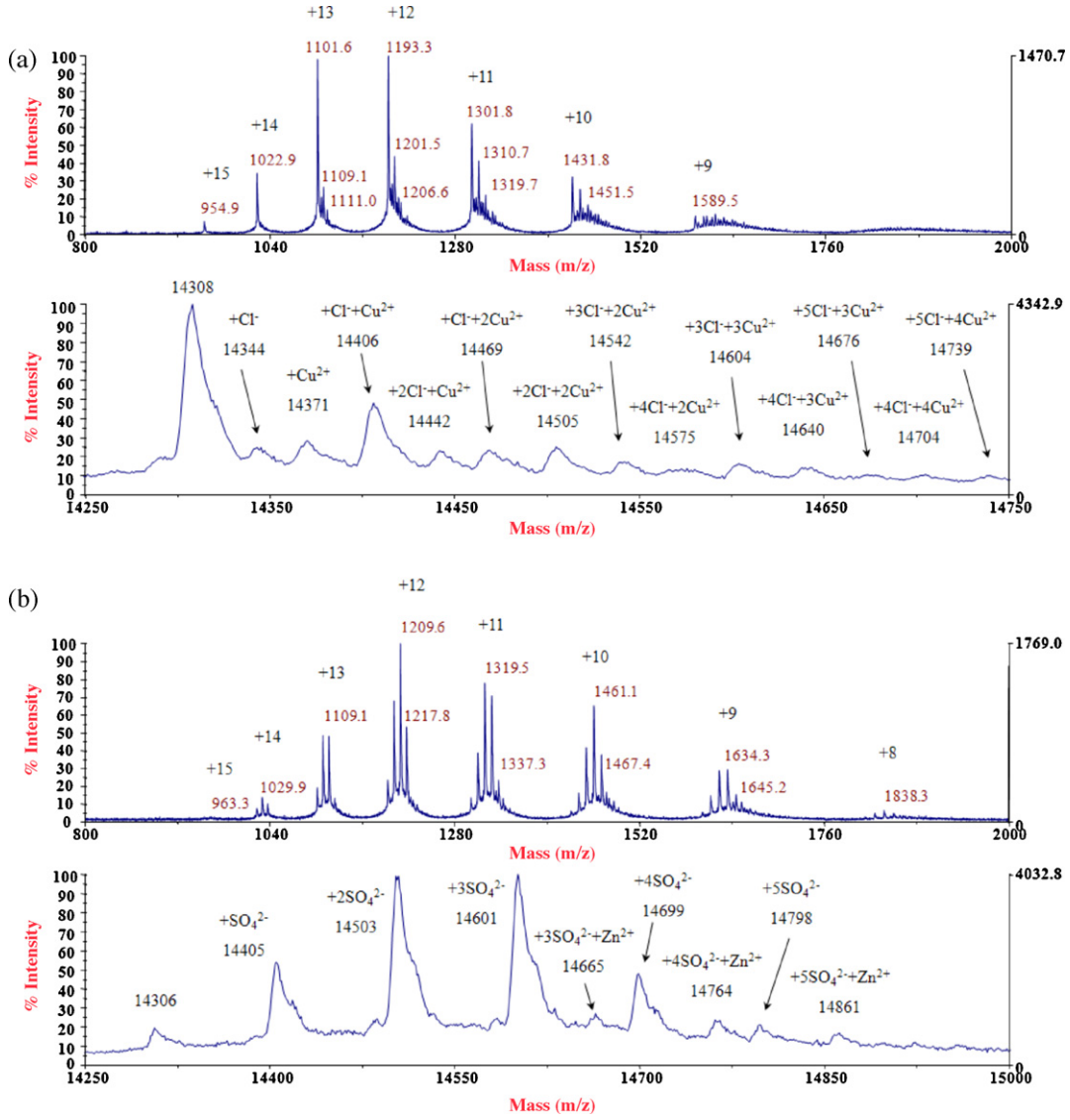


Fig. 2. ESI mass spectra of 10 μM Lys under addition of $CuCl_2$ in a 75-fold molar excess of Cu^{2+} against Lys (a), and of $ZnSO_4$ in a 20-fold molar excess of Zn^{2+} (b) in ACN/H_2O/HAc (49.5:49.5:1, v/v/v). ESI conditions: spray tip potential, 4000 V; nozzle potential, 120 V; 10 spectra accumulated. The lower spectra show the mass deconvolutions.

Also the use of metal nitrates led to problems in the spectrum interpretation. The nitrate ion has a very similar molecular mass to the Cu^{2+} ion. The complexation of one Cu^{2+} ion by a protein results in a mass increase of 62.6 g mol^{-1}, whereas the association of one nitrate ion leads to an increase of 62.0 g mol^{-1}. Mass spectra obtained from Lys after incubation with the nitrate salts of Zn(II) and Cu(II) in a molar ratio of metal:Lys = 75:1 exhibited the binding of both two nitrate ions and two metal ions in each case. The advantage of using nitrate salts is that very high peak intensities occur. The signal intensity of Lys in presence of Zn(II) nitrate in a 75-fold molar excess was about twice as high as the intensity under addition of Zn(II) sulfate in a 20-fold molar excess. In the majority of measured molar ratios of metal salts to Lys, the stoichiometry of the Cu–Lys complex was about 1 higher than that of the Zn–Lys complex (Table 3). For some samples, same stoichiometries were measured for Cu- and Zn-interactions with Lys. In most α-Ltb and Cyt c samples, the same number of Cu and Zn ions was bound to the proteins. Similar to Lys, more Cu ions than Zn ions were associated to α-Ltb and Cyt c in a few samples.

An influence of the reducing agent TCEP used during the protein preparation process from plant tissues [6] on protein metal interactions could not be observed by ESI-MS. In Fig. 3, the deconvoluted ESI mass spectra of Lys without and with addition of TCEP are compared. In both cases the binding of one Zn^{2+} ion to the protein was detected. Similarly, concerning the interaction of α-Ltb, Cyt c, and BSA with Zn and Cu ions, no effects of TCEP were found in the ESI mass spectra. This result indicates that reduced disulfide bridges do not contribute to the number of Cu and Zn binding sites of the proteins considered in these ESI-MS studies.

Mass spectrometric measurements of the proteins under different pH conditions revealed that the number of protein bound metal ions increased with increasing pH value of the sample solution (Table 3). This effect is ascribed to deprotonation of metal complexation sites of proteins and was observed also previously for Cu and Zn interactions with Lys [19]. From this pH effect can be concluded regarding the protein preparation from biological materials that a neutral or alkaline extraction buffer should be used to preserve metal–protein bindings.

The interpretation of the deconvoluted ESI mass spectra of BSA was more difficult than for the other proteins because only one broad peak resulted (Fig. 4). To find out how many metal ions are bound to this large protein ($M \approx 67$ kDa [15]), the molecular mass of BSA recorded without metal ion addition was subtracted from the mass of the protein measured after incubation with Zn or Cu ions. This mass difference resembles the molecular masses of all protein bound metal ions as well as salt anions. The number of metal ions bound to BSA could not be determined exactly on this way because it was not known how many anions like chloride or sulfate are simultaneously bound to the protein. The molecular mass of BSA examined in presence of Zn or Cu salts increased with increasing pH value indicating a higher number of metal ions bound to BSA as discussed above for the smaller proteins.

3.2. Influences of the GE protein preparation procedure on Zn and Cu bindings of model proteins

The binding capacities of Lys for Zn and Cu ions provided in different concentrations and in form of different salts were determined after performing a complete protein preparation procedure (see Fig. 1) including extraction, protein precipitation and washing of the protein pellet (Fig. 5). With an increasing molar excess of zinc in the original protein solution the binding capacity of Lys for Zn^{2+} ions increased (Fig. 5a). A similar behavior was observed in case of spiking of the simulated protein extract with Cu^{2+} ions, except for higher metal concentrations. Same binding capacities of Lys for Cu^{2+} provided in a 50- and 100-fold molar excess were determined (Fig. 5b), which leads to the assumption that the maximal binding capacity of Lys for Cu^{2+} ions was reached under these conditions. At the example of a 50-fold molar Zn excess, the effect of the salt anion becomes apparent and confirms previous results about the Zn binding on a complex plant protein mixture [6]. In spite of the buffered protein solution, the binding capacity for Zn correlates inversely with the acid strength of the anion. Also the ESI-MS binding studies demonstrated that the largest number of metal ions was bound to Lys if their acetate salts were applied (Table 3). If Cu(II) salts were used for spiking (Fig. 5b) of model protein extracts, this order was slightly modified. The highest binding capacity of Lys was reached for the addition of Cu(II) sulfate followed by acetate, whereas the lowest binding capacity resulted for Cu(II) chloride. Altogether, an about three times higher Cu binding capacity of Lys subjected to the GE protein preparation procedure was ascertained compared to the Zn binding capacity (compare

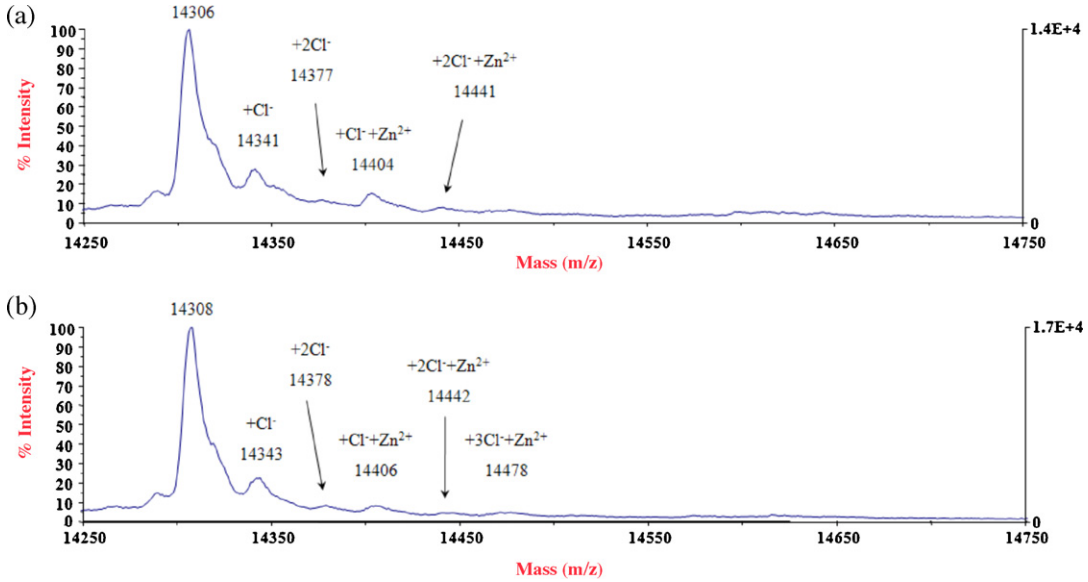


Fig. 3. Deconvoluted ESI mass spectra of 10 μM Lys under addition of $ZnCl_2$ in a 20-fold molar excess of Zn^{2+} against Lys without addition of TCEP (a) and with TCEP in a concentration of 40 μM (b) in ACN/H_2O/HAc (49.5:49.5:1, v/v/v). ESI conditions see Fig. 1.

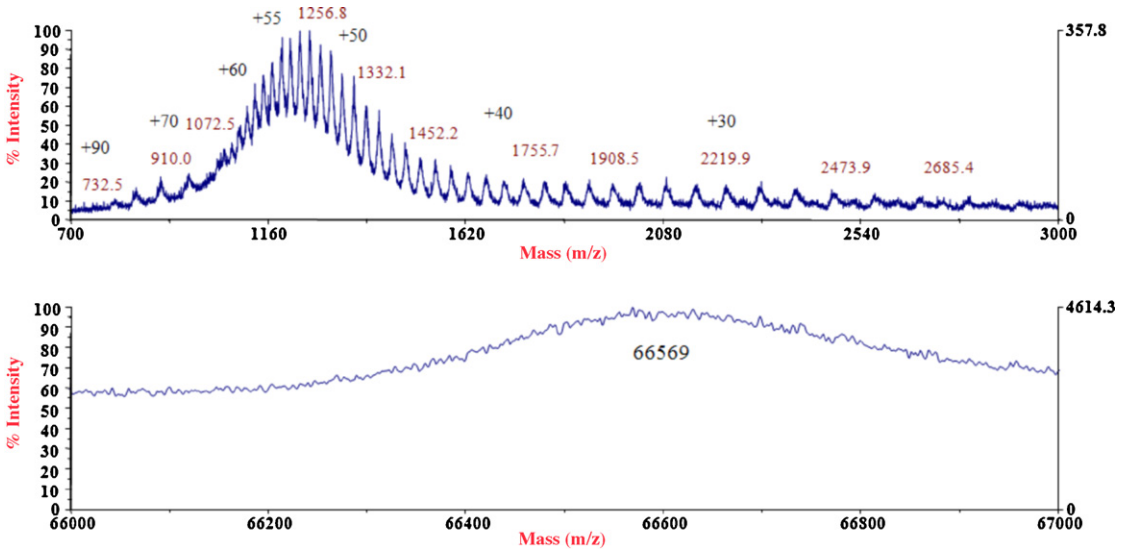


Fig. 4. ESI mass spectra of 10 μM BSA under addition of $ZnCl_2$ in a 20-fold molar excess of Zn^{2+} against the protein in ACN/H_2O/HAc (49.5:49.5:1, v/v/v). ESI conditions: see Fig. 1. The lower spectrum shows the mass deconvolution.

Fig. 5a and b). The binding of more Cu than Zn ions to Lys was also confirmed by the mass spectrometric binding studies (see Section 3.1, Table 3). However, binding stoichiometries resulting from the protein preparation procedure remain under those determined by the ESI-MS measurements (compare Fig. 5 and Table 3) even though same metal/protein ratios were examined with both methods. From this finding a great metal loss during the protein preparation steps especially when performing TCA precipitation can be deduced in comparison to the direct ESI-MS measurements.

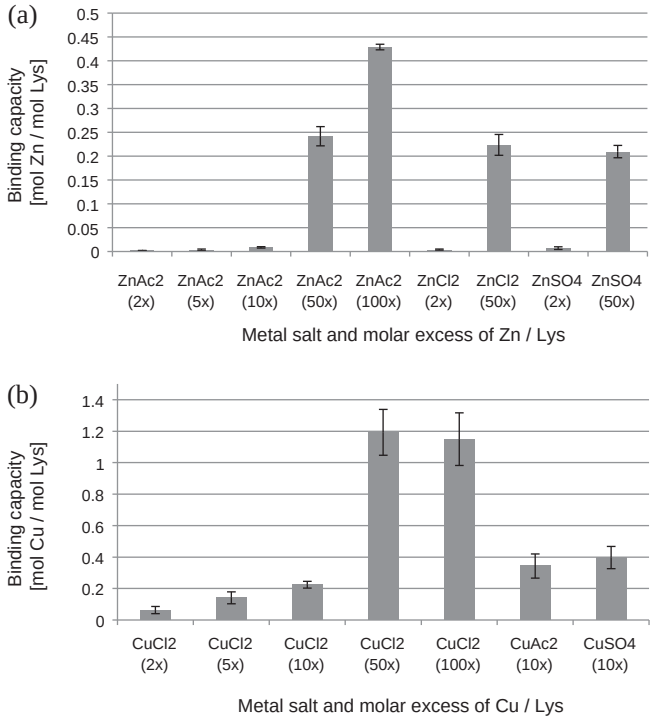
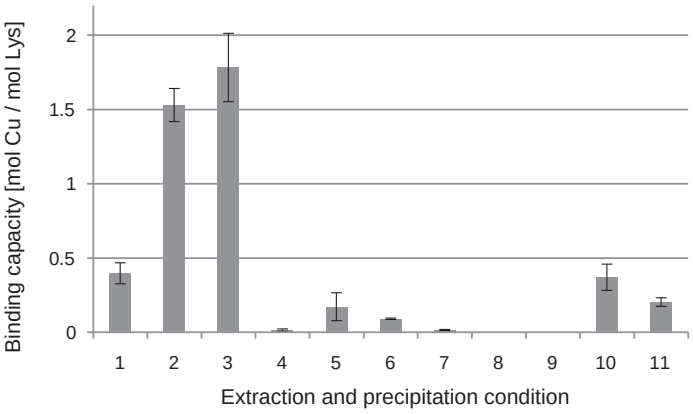


Fig. 5. Binding capacities of Lys for Zn (a) and Cu (b) added to a simulated protein extract in form of acetate, chloride, or sulfate in different concentrations. Composition of the extraction buffer: 5 mM Tris–HCl, pH 8.0, 1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF; protein precipitation: 20% (m/v) TCA in water. Average values with standard deviations from three parallel experiments are shown.

Both the composition of the extraction buffer and the concentration of the precipitating agent influenced the protein–metal interactions to a great extent. An increasing pH value of the extraction buffer led to an increasing Cu binding capacity of Lys (Fig. 6). This result was expected and also proven by the ESI-MS measurements (Section 3.1) because at a higher pH value more amino acid side chains are deprotonated, so that metal ions can coordinate to them. Interactions of both Cu^{2+} and Zn^{2+} ions are described with cysteine [20], histidine [21], glutamic acid and aspartic acid [22,23]. Furthermore, Cu^{2+} ions interact with methionine [24], whereas Zn^{2+} ions bind also to glutamine and asparagine [22]. All extraction buffer additives except for SDS exerted a positive effect on protein metal interactions. The omission of TCEP, PMSF, or PVPP led to a diminished extent of metal–protein binding. In case of TCEP, this finding disagrees with the ESI-MS measurements, where no effects of the reducing agent on metal–protein interactions were observed in the mass spectra (Section 3.1). Possibly, reducing conditions maintained during the protein preparation procedure prevent protein aggregation and therewith masking of metal binding sites. Besides, a greater molar excess of TCEP to the protein was applied during the protein preparation process (14-fold) than in the ESI-MS measurements (4-fold). Larger TCEP concentrations could not be measured by ESI-MS due to a strong ionization suppression of the protein by TCEP ion formation. The positive effects of the protease inhibitor PMSF and of the polyphenol adsorbens PVPP remain unexplained because the model protein solution contained both no proteases and no phenolic compounds. A possible overestimation of the metal binding capacity caused by PVPP was corrected by the PVPP blank value (see Section 2.2). But from these results can be concluded that no inhibitions of metal–protein interactions owing to PMSF or PVPP additives must be considered for protein extraction from real biological materials. The tenside addition to the extraction buffer which enhances the extraction efficiency of hydrophobic proteins from cells, inhibited the Cu binding to Lys nearly completely due to an inclusion of the protein molecules inside of micelles. A similar negative effect caused by SDS was observed for arsenic bindings to plant proteins [12]. At this point it is assumed that the sequence of the tenside and metal addition to the protein solution must be considered. In the current experiments, the micelles were formed before the proteins were incubated with the metal salt. However, if proteins were extracted from biological tissues, their metal bindings were formed before the



Extraction and precipitation conditions:

Nr.	Extraction buffer	Extraction buffer additives	Precipitating agent
1	5 mM Tris-HCl, **pH 8.0**	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF	**20 %** (m/v) TCA in water
2	5 mM Tris-HCl, **pH 10.0**	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF	20 % (m/v) TCA in water
3	5 mM Tris-HCl, **pH 12.0**	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF	20 % (m/v) TCA in water
4	5 mM Tris-HCl, pH 8.0	1 g L^{-1} PVPP, 2 mM PMSF	20 % (m/v) TCA in water
5	5 mM Tris-HCl, pH 8.0	1 g L^{-1} PVPP, 2 mM TCEP	20 % (m/v) TCA in water
6	5 mM Tris-HCl, pH 8.0	2 mM TCEP, 2 mM PMSF	20 % (m/v) TCA in water
7	5 mM Tris-HCl, pH 8.0	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF **0.5% (m/v) SDS**	20 % (m/v) TCA in water
8	5 mM Tris-HCl, pH 8.0	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF **1% (m/v) SDS**	20 % (m/v) TCA in water
9	5 mM Tris-HCl, pH 8.0	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF **2% (m/v) SDS**	20 % (m/v) TCA in water
10	5 mM Tris-HCl, pH 8.0	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF	**10 %** (m/v) TCA in water
11	5 mM Tris-HCl, pH 8.0	1 g L^{-1} PVPP, 2 mM TCEP, 2 mM PMSF	**30 %** (m/v) TCA in water

Fig. 6. Influences of composition and pH value of the extraction buffer and of the TCA concentration of the precipitating agent on binding capacities of Lys for Cu added as Cu(II) sulfate in a 10-fold molar excess to the protein. Average values with standard deviations from three parallel experiments are shown.

exposure to SDS so that the whole metal–protein complexes can be included into the micelles. With an increasing concentration of the strong acid TCA in the precipitating solution, the binding capacity of Lys for Cu^{2+} ions decreased due to competing H^+ binding.

A strong influence of the type of the precipitating agent on the metal binding capacity of the model proteins Lys and BSA was ascertained (Fig. 7). Most metal ions were bound to both proteins which differ in size and p*I* value if acetone precipitation was carried out: up to 15 Zn ions or 13 Cu ions bind to one Lys molecule whereas up to 76 Zn and 78 Cu ions, respectively, coordinate to BSA. This enhanced binding stoichiometry for Zn and Cu–Lys complexes exceeds the stoichiometric ratios of metal–protein complexes determined by ESI-MS (Section 3.1) and also the Zn binding capacity of Lys determined in the extraction buffer by means of ultrafiltration (see below, Section 3.4, left bar in Fig. 8). This indicates that the extent of Zn binding by the proteins is enhanced during the acetonic precipitation compared to the binding state of proteins dissolved in the extraction buffer before precipitation. The lowest binding capacity occurred by using TCA as precipitating agent. As mentioned above, this results from the very low pH value of the TCA solution. A strongly diminished arsenic binding capacity of plant protein fractions resulted after performing TCA precipitation instead of acetone precipitation in a previous study [6]. After ammonium sulfate precipitation, the binding capacity of both proteins was enhanced compared to TCA treatment but remained small compared to acetone precipitation. In order to precipitate the larger BSA, a higher $(NH_4)_2SO_4$ concentration (70% m/v) was necessary than for the smaller Lys (20% m/v). A further advantage of using ammonium

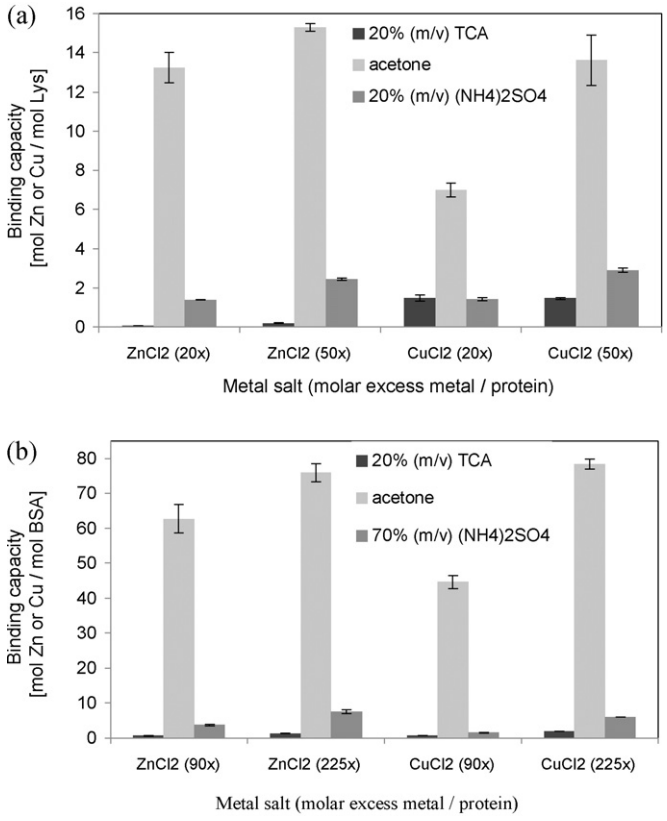


Fig. 7. Zn and Cu binding capacities of Lys (a) and BSA (b) resulting due to addition of the metal chlorides in different molar excess and in dependence on the precipitating agent. Protein preparation procedure: Extraction buffer: 5 mM Tris–HCl, pH 8.0, 2 mM TCEP, 2 mM PMSF. Average values with standard deviations from three parallel experiments are shown.

sulfate or acetone is that the proteins were not denatured during precipitation (acetone must be slowly added), whereas TCA causes an irreversible protein denaturation. Unfortunately, greater resolution and sharpness of 1D gels from plant proteins were obtained after TCA precipitation compared to acetone precipitation [6]. The Cu and Zn binding capacities of Lys resulting from both TCA and $(NH_4)_2SO_4$ precipitation remained under those ascertained in the starting extraction buffer before protein precipitation (compare Fig. 7a with left bar in Fig. 8 for application of $ZnCl_2$ in a 20-fold molar excess of Zn to Lys). The Lys binding capacities for Cu and Zn were unequally influenced by the precipitation step. Whereas a higher binding capacity of Lys for Cu than for Zn was recorded both in ESI-MS binding studies (Section 3.1), after TCA precipitation (Figs. 5 and 7) and in the different GE buffer systems in ultrafiltration experiments (see below, Section 3.4), the acetonic treatment of the protein enhanced the Zn binding capacity compared to the Cu binding (Fig. 7).

Because acetone proved to be the most-preserving precipitating agent concerning the stability of metal–protein bindings it was chosen as washing agent for purification of the protein pellet obtained from the precipitation step (Fig. 1). The Zn loss occurring during this washing step was quantified in our previous work for plant proteins [6] and amounted to $21.2 \pm 5.5\%$ ($n = 3$) of the Zn content of the original pellet before washing.

3.3. Metal loss by dialysis of the resolubilized protein

A desalting step for the precipitated, washed, and resolubilized protein proved to be essential to achieve the focusing voltage during 1st dimension of 2D-GE of plant samples [6]. However, especially coordinative metal–protein bindings are expected to dissociate during the dialysis procedure due to the osmotic gradient. A comparison between the metal concentrations in protein samples resolubilized for 2D-GE before and after dialysis showed that the desalting step led to a loss of protein bound metal ions between 10 and 35% of the starting concentration before dialysis. For an initial 50-fold molar excess of Cu to Lys, the precipitation with TCA led to a smaller metal loss in the dialysis procedure ($10.2 \pm 4.9\%$, $n = 3$) than the precipitation with ammonium sulfate ($34.5 \pm 4.7\%$, $n = 3$) and with acetone ($20.9 \pm 2.4\%$, $n = 3$). A similar Zn loss of acetonically precipitated Lys was noticed ($22.4 \pm 3.4\%$, $n = 3$). The higher metal loss of the protein precipitated with acetone compared to the TCA-precipitated protein can be possibly ascribed to its higher metal contents (compare data in Fig. 7) comprising also a larger part of unspecifically bound metal ions which can be easily removed by dialysis. For BSA incubated with a 225-fold metal excess and precipitated with acetone, $15.0 \pm 2.5\%$ ($n = 3$) and $14.8 \pm 4.0\%$ ($n = 3$) of the Cu and Zn concentration, respectively, in the resolubilized protein sample were removed by dialysis. This finding indicates a stronger metal ion binding to BSA in comparison to Lys.

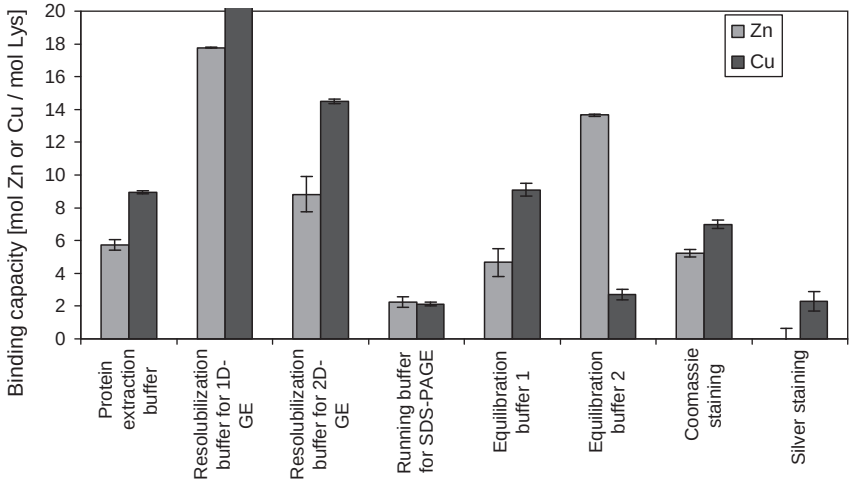


Fig. 8. Binding capacities of Lys for Zn^{2+} and Cu^{2+} in different buffer systems applied during 1D- and 2D-GE, determined by ultrafiltration over size exclusion membranes. Compositions of the buffer systems see Table 1. Average values with standard deviations from three parallel prepared samples are shown or a metal/Lys ratio of 20:1 before ultrafiltration.

3.4. Examination of the influences of 1D- and 2D-GE buffer systems on metal–protein-bindings by means of ultrafiltration

Because most components of the protein sample preparation for GE as well as of the GE running buffers and staining solutions possess no compatibility with the electrospray ionization, their effects on metal–protein interactions cannot be investigated by using ESI-MS. But the ultrafiltration of protein solutions containing the mentioned GE components over size exclusion membranes offers a good possibility to separate unbound metal fractions from the protein pool and therewith to quantify the metal losses from the proteins caused by the different chemicals. A pronounced dependence of the binding stoichiometry of Cu and Zn-Lys complexes on the composition of the buffer solutions was found (Fig. 8). Because the highest binding capacity was achieved in the resolubilization buffer for 1D-GE, the components of the 1D-GE resolubilization buffer (62.5 mM Tris buffer, 2% (m/v) SDS, 5% (v/v) β-mercaptoethanol, 20% (v/v) glycerole, 0,1% (m/v) bromophenol blue) do not or scarcely impede protein metal interactions in comparison to the other tested buffer systems. In contrast to this, 0.5–2% (m/v) SDS in the protein extraction buffer hindered the metal binding to Lys if the protein was subjected to the complete protein preparation procedure (see Section 3.2). An error source of the ultrafiltration experiments could be that glycerole partially plugs the pores of the UF membranes because the filtrate volumes obtained from the 1D-GE resolubilization buffer were slightly reduced compared to the other buffer solutions.

Because denaturing conditions are assumed to destroy coordinative metal–protein bindings, non-denaturing 1D-GE variants in which no SDS is employed are favored for the analysis of metal-binding proteins [5,9,25]. However, instabilities of Cu and Zn–protein associations were also observed during native GE separations [8] since the running buffers contain metal-complexating substances such as glycine or tricine that break coordinative protein–metal bindings (see below).

In the resolubilization buffer for 2D-GE less metal ions are bound to Lys than in the 1D-GE buffer but more than in the extraction buffer. The urea component of the 2D-GE resolubilization buffer decreased the affinity of Lys for Cu^{2+} ions as already been found by Hutchens and Yip [26]. To avoid the urea component that is needed for the conventional IEF, a native 2D-GE protocol was proposed for metalloproteome analysis [27].

It remains unclear why the extraction buffer that contains only tris, TCEP, and PMSF and not such more protein-structure changing components like urea and the tensides SDS or CHAPS caused a lower metal binding capacity of Lys in comparison to both GE resolubilization buffer systems. Possibly, the protein is more defolded in these solutions so that more metal binding sites are exposed to the protein surface.

The running buffer of 1D-GE exerted a very negative effect on protein metal interactions. An about eight times lower binding capacity of Lys for Zn^{2+} and Cu^{2+} ions was reached in this buffer system compared to the resolubilization buffer for 1D-GE. The reason for this can be ascribed to the running buffer component glycine which can bind Zn^{2+} and Cu^{2+} ions as bidentate ligand [23]. Glycine appears as free glycinate ion at a pH of 8.8 prevailing in the running buffer [8], so that it can form complexes with metal cations like Zn^{2+} and Cu^{2+}. A dissociation of protein bound metal ions during 1D-GE was observed by Añorbe et al. [4].

The equilibration buffers 1 and 2 which were applied to the proteins between IEF and PAGE during 2D-GE only differ by one substance. While the first buffer contains DTT, in the second buffer iodoacetamide is applied. Despite a differing composition, very similar metal binding capacities were achieved in equilibration buffer 1 and in the extraction buffer. Further, in the first equilibration buffer a higher binding capacity for Cu^{2+} ions than for Zn^{2+} ions was observed than in the second buffer, whereas in the second buffer more Zn^{2+} ions than Cu^{2+} ions bind to the protein. Since both buffer systems contain several chemicals, responsible effects are difficult to interprete. For example, the coordination of heavy metal ions by DTT [28] can compete with the protein binding.

Both Coomassie staining and silver staining diminish the binding capacity of Lys for metal ions. Molecules of Coomassie brilliant blue bind unspecifically to the protein and possibly block the binding sites for metal ions sterically. At this point must be stated that also the blue native polyacrylamid gel electrophoresis (BN-PAGE) [27,29] holds a danger for dissociation of metal–protein complexes since the protein-binding dye Coomassie brilliant blue G 250 replaces the tenside SDS. Raab et al. [9] explain the loss of protein bound metal ions during Coomassie staining with the presence of phosphoric acid in the staining solution. The strongly negative effect of silver staining on protein metal interactions presumably results from a competitive binding of Ag^{+} ions to sulfhydryl groups of the protein [23].

In case of most buffer systems examined by ultrafiltration, a higher binding capacity was calculated from the difference of the filtrate concentrations and the original metal spiking amounts in the protein solutions than the complex binding stoichiometry that was measured by ESI-MS. From the ultrafiltration experiments as well as from the microwave-digested protein pellets of the GE sample preparation procedure a summarized binding stoichiometry is obtained that is not resolved from the position of the binding equilibrium because unbound and metal-bound protein molecules are not separated from each other. Therefore, the term binding capacity should be used instead of binding stoichiometry which can be deduced from the ESI mass spectra.

4. Conclusion

Metal–protein binding studies, which were performed with three independent methods, allowed an evaluation of the influences of sample preparation and GE running parameters on the metal binding capacities of some model proteins. Nearly all buffer systems as well as individual components of them used in the GE process changed the metal binding state of the proteins. Especially an SDS containing protein extraction buffer, the 1D-GE running buffer, and gel-staining solutions suppressed the metal binding capacities of lysozyme. Acetone can be recommended as precipitating agent whereas TCA and ammonium sulfate strongly diminish the metal binding capacity of produced protein fractions. In the ESI-MS measurements, the acetate salts of the metals led to the highest binding stoichiometries of metal/protein complexes compared to the chloride, sulfate, and nitrate salts. Furthermore, in contrast to the other mentioned salt anions, no acetate–protein adducts occurred in the mass spectra.

The problem of the approach pursued in the current study exists in the lack of a standard protein sample which reflects the native binding state of the proteins and their affinity for metal ions under physiological conditions. Therefore, all results obtained by the ESI-MS and ICP-MS measurements remain at a relative state. Furthermore, metals have been spiked to the proteins at the beginning of the experiments, and the behavior of metal–protein binding dissociations could be different in a real system. Nevertheless, valuable conclusions can be deduced concerning the design of 1D- and 2D-GE experiments for investigations of metal-binding proteins.

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