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Metal–protein binding losses in proteomic studies by PAGE–LA-ICP-MS

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ABSTRACT

Some experiments to study the influence of electrophoresis conditions and subsequent LA-ICP-MS (laser ablation-inductively coupled plasma mass spectrometry) determination of two metal-binding proteins with different metal–protein affinities (superoxide dismutase, containing Cu and Zn, and alcohol dehydrogenase, containing Zn) are performed. In metal-binding proteins with weak metal–protein affinities, metal losses can happen during electrophoretic separation. It has been demonstrated that the detection of these metals bound to the proteins depends, not only on the nature of the electrophoretic process (naturing or non-denaturing) and post-separation gel treatment, but also on the trailing ion chosen and current applied in the electrophoretic method used. Non-denaturing methods are preferred to denaturing ones in the case of alcohol dehydrogenase being BN-PAGE (Blue Native-Polyacrylamide Gel Electrophoresis) with the use of Tricine as trailing ion the most recommended method. The concentration obtained for Zn in ADH applying BN-PAGE–LA-ICP-MS was $2.6 \pm 0.30\ mg\ g^{-1}$ very close to the one obtained for ADH solution by ICP-MS ($3 \pm 0.23\ mg\ g^{1}$). For superoxide dismutase either denaturing or non-denaturing electrophoresis conditions can be used, but a denaturing method based on the use of Tricine as trailing ion is recommended to preserve metals–protein binding when the use of non-denaturing conditions must be avoided. The found concentration for Cu and Zn in SOD after applying SDS–Tris–Tricine-PAGE–LA-ICP-MS was 2.5 ± 0.33 and $2.4 \pm 0.37\ mg\ g^{-1}$ respectively, more or less close (especially for Cu) to the one obtained in SOD solution by ICP-MS (3 ± 0.21 and $3.7 \pm 0.32\ mg\ g^{-1}$ for Cu and Zn). We observe that as higher current is applied the possibility of metal–protein binding losses is higher. In all cases staining of the gel prior to LA-ICP-MS is not recommended.

1. Introduction

During the last few years, laser ablation (LA) inductively coupled plasma (ICP) mass spectrometry (MS) has been used increasingly as a detection system of metals in proteins after separation by polyacrylamide gel electrophoresis (PAGE) using both one-dimensional (1D) PAGE [1–17] and two-dimensional (2D) PAGE [4,6,11,14,18–29]. This procedure has become established as very efficient technique for proteomics studies in life sciences.

In metalloprotein speciation is very important to maintain the integrity of metal–protein binding during all the process. In most of the applications using PAGE–LA-ICP-MS, the subject of the study was heteroatom-containing proteins, such as selenoproteins [1,4,6,7,10,11,19,28] and phosphoproteins [2,3,18,20,21,23,24,27], where the elements are part of the proteins and are strongly bound to them, so the binding is not broken during electrophoresis separation. However, metal-binding proteins in which metal–protein interactions are of lower affinity can cause loss of the metal during electrophoresis separation, especially using denaturing PAGE. Because of this, the main pitfall, which researchers have to face, is that it is not always possible to keep these metalloprotein complexes intact during separation and post-separation process, and metal losses can occur. So, it is necessary to develop new strategies to separate proteins without breaking the metal–protein binding. Some authors have already discussed these metal losses in different proteins, and in most cases the use of native-PAGE instead of denaturing PAGE, has been proposed as solution to the problem.

McLeod's group [3,6] studied Pt-serum binding proteins subject to LA-ICP-MS after both native- and denaturing-PAGE. While after native PAGE representative Pt signals were obtained for these proteins, no signals were observed after denaturing PAGE.

Chery et al. [31] found vanadium losses of serum proteins during denaturing electrophoretic separation, therefore the use of native PAGE, such as Blue Native (BN)-PAGE, where all denaturing agents are left out of the gel electrophoresis, was necessary to avoid vanadium losses.

Becker et al. separated yeast mithocondrial proteins [21,24] by 2D isoelectricfocusing (IEF)/sodium dodecyl sulfate (SDS)-PAGE and human brain proteins [22,23] by 2D BN/SDS-PAGE and analyzed the gels by LA-ICP-MS. They demonstrated that stable metal–protein complexes can survive denaturing and reducing conditions during the separation of proteins in the second dimen-

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sion, but also that these conditions partly releases the naturally bound metal ions of non-stable metal-complexes. According to these authors, the most challenging problems are related to the preservations of the naturally bound metal ions during gel separation. In recent papers they have also described the use of BN-PAGE to study the binding of Cu and Zn to albumin [14], and to analyze some metals such as Cu, Zn and Fe in rat tissues proteins [15]. Moreover, to study the same metals in rat tissues, they have described a native 2D BN-PAGE in order to keep the metal complexes intact during separation [29].

Jakubowski et al. have investigated the detection of Cd, Zn and Fe in different metalloproteins by LA-ICP-MS after both anodal native PAGE and denaturing PAGE [9,12]. The results showed that denaturing PAGE was unsuitable for quantitation of the metals in these proteins because of the possible metal losses by the use of it, especially in weakly bound metal containing proteins. However, anodal native PAGE, which preserves the native protein structure, was a powerful alternative but has to be paid at the cost of molecular weight calibration.

However, not only the nature of the electrophoretic process (denaturing or non-denaturing) can alter the metal–protein binding. There are few studies in relation to the influence of other electrophoresis conditions (including post-separation process) on possible metal–protein bindings break. Gao et al. [30] found that not only the influence of the electric field during electrophoresis separation, but also the processes of fixing, staining and destaining of proteins in gel may result in loss of metal ions from metallopoteins in human liver cytosol using synchrotron radiation X-ray fluorescence as detection system after SDS-PAGE.

In a previous paper we studied the behaviour of two metalloproteins (superoxide dismutase (SOD), containing Cu and Zn, and thyroglobulin, containing I) and metal-dissolved organic matter (DOM) complexes after PAGE–LA-ICP-MS [16]. Denaturing conditions allowed determining I in thyroglobulin, because I is strongly bound to the protein, while it was not possible to detect Cu and Zn in SOD in the same conditions due to metal losses under these conditions. We proposed the replacing of Glycine by Tricine as trailing ion instead of using the conventional native PAGE. The influence of current applied in the speciation of metal–DOM complexes from a real compost sample by PAGE–LA-ICP-MS was also demonstrated.

In a very recent paper, Feldmann and coworkers [17] have evaluated the gel electrophoresis conditions for the separation of metal-tagged proteins by GE-LA-ICP-MS, focusing on the stability of metal–protein binding during GE and post-separation methods. They found that the most suitable gel electrophoretic techniques studied to separate metal–protein complexes and later detection of metals by LA-ICP-MS are the native ones. They also found that staining of the gel prior to LA-ICP-MS is not recommended, since most protein-bound metal is lost during the staining procedure (except with covalently bound), and should be avoided prior to ablation. Furthermore, they proposed line scanning better than raster spot analysis to avoid misinterpretation due to contamination in/on inhomogeneous gels.

The aim of this paper is to study how different electrophoretic conditions may influence the separation of metal–proteins by PAGE–LA-ICP-MS in relation to stability of metal–protein binding. Not only the nature of the electrophoretic process and the staining and destaining procedures, but also the trailing ion chosen, and current applied in the electrophoretic method used. We have applied this study to superoxide dismutase (SOD) and alcohol dehydrogenase (ADH). SOD consists of two units, each one with a molecular weight of 16,250 Da containing Cu and Zn. ADH is supposed to be a 141,000 Da tetramer containing four equal subunits (35.25 kDa) whose active sites contain Zn. SOD is considered to be a metalloprotein (where metals bind to proteins with high-affinity interactions) [32]. Metals such as Zn are generally non-covalently complexed to proteins.

2. Experimental

2.1. Instrumentation

Gel electrophoresis was carried out with a MiniProtean® 3 electrophoresis cell connected to a PowerPac™ basic power supply (Bio-Rad Laboratories, Hercules, CA, USA). The gels were vacuum dried prior to LA-ICP-MS measurements with a model 583 gel dryer (Bio-Rad Laboratories, Hercules, CA, USA).

A Nd-YAG LA system operating at 213 nm (UP-213, New Wave Research, Huntington, UK) was coupled to an ICP ion source mass spectrometer (Perkin Elmer Elan DRC-e, Toronto, Canada). Prior to all experiments, the ICP-MS instrumental and LA set-up were optimized as described elsewhere [33]. Typical operating parameters for LA-ICP-MS are summarized in Table 1. More experimental details in relation to LA-ICP-MS system are given in a recent paper [16]. For each protein, each PAGE lane was completely and continuously scanned by the laser beam in order to study possible metal losses in different parts of the gel. The ablated material was transported by Ar as carrier gas into the ICP. The isotopes monitored included the following ^{13}C, ^{63}Cu, ^{65}Cu, ^{64}Zn and ^{66}Zn. ^{13}C was used as an internal standard for correcting signal drift.

2.2. Reagents, standards and samples

Tris(hydroxymethyl)aminomethane (Tris), sodium dodecyl sulfate (SDS), Glycine, N-tris(hydroxymethyl)methylglycine (Tricine), ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), 30% 29:1 acrylamide/biscrylamide solution (3.3% C), Coomassie® Brilliant Blue R-250, and Coomassie® Brilliant Blue G-250 of electrophoresis purity were purchased from Bio-Rad Laboratories (Hercules, CA, USA). 2-Mercaptoethanol was provided by Fluka BioChemika (Buchs, Switzerland). Bromophenol blue was from Doesder (Barcelona, Spain). Methanol and acetic acid for analysis were from Poch (Gliwice, Poland). Glycerol was from Probus (Barcelona, Spain). HCl, HNO_3, in-stock solution ($1000\,mg\,L^{-1}$) and trace metal standard I solution ($100\,mg\,L^{-1}$) were purchased from J.T. Baker (Phillipsburg, NJ, USA). H_2O_2 was

Table 1
Instrumental operating conditions and measurement parameters for laser ablation inductively coupled plasma mass spectrometry (ICP-MS).

ICP-MS	
Forward power (W)	1000
Nebulizer Ar gas flow rate	$0.95\,L\,min^{-1}$ (for liquid introduction)
Coolant Ar flow	$14\,L\,min^{-1}$
Sweeps/reading	1 (20)[a]
Readings/replicate	According to laser scan line length (1)[a]
Replicates	1 (3)[a]
Dwell time	50 ms
Acquisition mode	Peak hopping
Detector mode	Dual/pulses (counting and analog)
Autolens	On
Isotopes monitored	^{13}C, ^{63}Cu, ^{65}Cu, ^{64}Zn, ^{66}Zn
UP-213 laser system	
Light source	Ring
Laser energy	60% ($10\,J\,cm^{-2}$)
Repetition rate	20 Hz
Size of laser beam	100 μm
Laser firing mode	Continuous (line)
Line scan speed	60 μm/s
Ar carrier gas flow rate	$1\,L\,min^{-1}$

[a] Conditions for determination of total metal content in proteins solution and in digested gels.

from Merck (Darmstadt, Germany). Water (18.2 MΩ cm^{-1}) was obtained from a Milli-Q system (Millipore, Bedford, MA, USA).

Albumin (from chicken egg white) (molecular weight 44.287 kDa), myoglobin (from horse heart) (molecular weight 16.949 kDa), and trypsin inhibitor (from Glycine max soybean) (molecular weight 20.000 kDa) were used as molecular weight standards. They were from Sigma–Aldrich Chemie (Stenheim, Germany).

The proteins used for the investigation were superoxide dismutase (SOD) (from bovine erythrocytes) and alcohol dehydrogenase (ADH) (from *Saccharomyces cerevisiae*) and were purchased from Sigma–Aldrich Chemie (Stenheim, Germany).

2.3. Gel electrophoresis

Proteins SOD and ADH were subjected to four different one-dimensional PAGE techniques.

2.3.1. Method 1 SDS–Tris–Glycine-PAGE

Proteins were separated by 1D denaturing gel electrophoresis performed on a discontinuous and vertical SDS gel according to Laemmli method [34] based on a Tris–Glycine system. Stacking gel was prepared at 4%, and resolving gel at 16%. Sample were dissolved 1:1 in 50 mM Tris buffer (pH 6.8) containing 2.4% SDS and 5% 2-mercaptoethanol and heated for 5 min at 100 °C in order to denature proteins prior to loading onto the gel. The gels were run at a current of 30 mA.

2.3.2. Method 2 SDS–Tris–Tricine-PAGE

Protein separation was carried out by 1D denaturing gel electrophoresis according to Schägger [35] based on a Tris–Tricine system. Stacking gel was prepared at 4%, and resolving gel at 12%. Prior to electrophoresis, proteins were denatured in 50 mM Tris (pH 6.8) buffer containing 4% SDS and 2% 2-mercaptoetanol at 100 °C for 5 min. To evaluate the current influence the gels were run at a current of 20 and 40 mA.

2.3.3. Method 3 Anodal Native-PAGE (AN-PAGE)

AN-PAGE was carried out with a discontinuous modified Ornstein and Davis [36] Tris/Glycine buffer system [9]. Stacking gel was prepared at 4%, and resolving gel at 6%. Samples were dissolved 1:1 with 600 mM Tris (pH 8.9) and loaded onto the gel. The gels were run at 20 mA for initial sample stacking; then, it was put at a current of 25 mA; and lastly, it was put at 30 mA.

2.3.4. Method 4 Blue Native-PAGE (BN-PAGE)

A BN-PAGE was performed to separate proteins according to the method of Schägger [37] based on Tris/Tricine buffer system. Stacking gel was prepared at 4%, and resolving gel at 10%. Prior to loading onto the gel, samples were mixed with a 50 mM Tris (pH 7.0) buffer containing 0.35% Coomassie Brilliant Blue G-250. Electrophoresis conditions consisted of a constant voltage of 60 V (current: 20–10 mA).

In all methods, the gels were run in duplicate. One gel was stained with a 25% methanol, 10% acetic acid solution containing 0.1% (w/v) Coomassie® Brilliant Blue R-250 and then washed in a 25% methanol solution overnight. Both gels were vacuum dried by the use of a gel dryer. Saran wrap was laid on the top of the gels to protect them from contamination until analysis by LA-ICP-MS. No problems with gels drying procedure were observed in relation to a successful detection of metals by LA-ICP-MS.

2.4. Analysis of the total metal content in proteins

The total metal content in each protein was analyzed by ICP-MS: Cu and Zn in SOD and Zn in ADH. SOD and ADH solutions were prepared by dissolving 2 mg of protein in water with appropriate dilution. External calibration was applied using metal standard solutions from 0 to 200 μg L^{-1}. ^{115}Indium was used as the internal standard to compensate for matrix effects. The content of Cu and Zn in SOD was respectively 3 ± 0.21 and 3.7 ± 0.32 mg g^{-1}. The content of Zn in ADH was 3 ± 0.23 mg g^{-1}.

The metal content in gels corresponding to proteins bands after electrophoresis was also determined in the gels where no metal losses were observed. Portions of gel corresponding to protein bands (around 10 mg) and blank gels (also around 10 mg) were digested with a mixture of 0.5 mL of nitric acid, 0.5 mL of hydrogen peroxide and 5 mL of ultrapure water (18.2 MΩ cm^{-1}) using a microwave digestion system (Mars 5, CEM, NC, USA). The digestion procedure consisted of two steps: (1) 400 W, 800 psi, 200 °C, 10 min ramp, 3 min hold and (2) 800 W, 800 psi, 200 °C, 0 min ramp, 6 min hold. After digestion, gel samples were diluted with ultrapure water. For quantification, ^{115}indium was used as the internal standard to compensate for matrix effects and external calibration was applied.

3. Results and discussion

3.1. Denaturing PAGE conditions

Initially denaturing conditions were used for separation of SOD and ADH with subsequent LA-ICP-MS detection.

A SDS–Tris–Glycine-PAGE method (method 1) was applied. The molecular masses of the proteins were estimated (17 kDa for SOD and 34.3 kDa for ADH) and resulted similar to the theoretical values (16.25 kDa for SOD and 35.25 kDa for ADH). After LA-ICP-MS measurements of both stained and unstained gels, there were no significant Cu and Zn signals in neither of the bands of SOD and ADH proteins in both stained and unstained gels (see Fig. 1 in which LA-ICP-MS signals of unstained gels are shown). PAGE lane scan is completely shown including stacking, and separating gel to see possible metal losses in different parts of the gel. In Fig. 1a, Cu and Zn signals are shown for SOD. Some ion signals appear in the stacking gel (maybe due to some contamination), but the highest Cu and Zn signals can be seen in the zone corresponding to the electrophoresis front. As we previously explained [16] we suspected that metals are lost from the protein during electrophoretic process maybe because they form complexes with Glycine trailing ion, when proteins enter the resolving gel and metals advance with the electrophoresis front. This fact is now confirmed in Fig. 1a, where there is a Cu and Zn peak at the end of the lane coinciding with electrophoresis front. At the beginning we thought that it could be some uncontrolled contamination, but no Cu and Zn signals were observed in blank lane in electrophoresis front position. The same results can be observed for Zn in ADH in Fig. 1b. This protein was not previously studied in our work [16]. It can be observed that there is a small Zn signal between stacking and resolving gels and a very small Zn peak corresponding to the band of protein, but in this case the most significant Zn peak also corresponds to electrophoresis front. Observing the blank lane signals for Zn there is some contamination in stacking gel, but no Zn signals appear in the zone corresponding to electrophoresis front for blank. As a consequence, we can say that using denaturing conditions with Glycine as trailing ion, Cu and Zn are lost by SOD and ADH in the electrophoresis process.

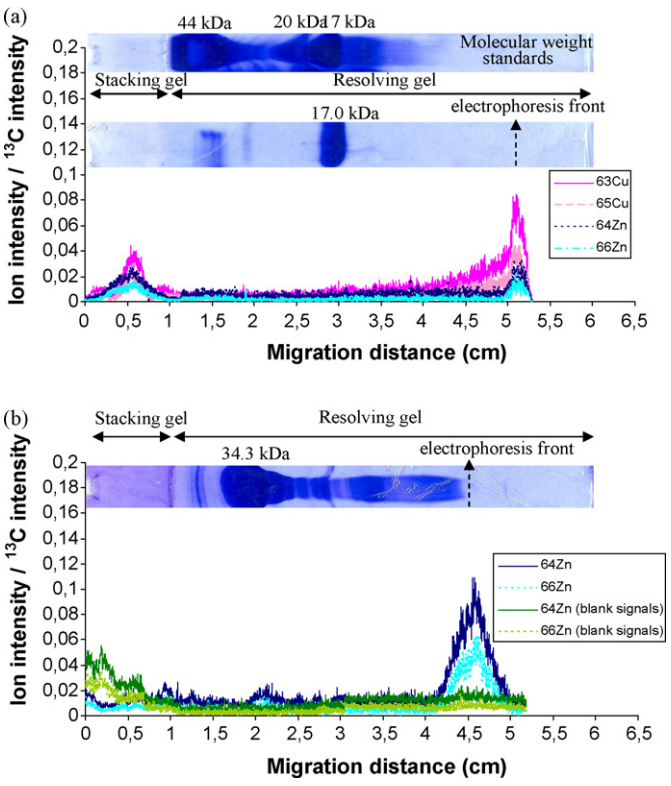

Fig. 1. Detection of metals in proteins by LA-ICP-MS after SDS–Tris–Glycine-PAGE (method 1) (unstained gels) (a) Cu and Zn in SOD and (b) Zn in ADH.

3.2. Effect of trailing ion

An alternative method (SDS–Tris–Tricine-PAGE, method 2), where Glycine was replaced by Tricine as trailing ion, was applied to avoid metal losses during electrophoresis. It was also suggested in our previous work [16] but now the current applied was less than the one used in the previous work (20 mA instead of 30 mA). Tricine is used for proteins with molecular mass below 14 kDa [37]. At the usual pH values, Tricine migrates much faster than Glycine and causes less protein movement. Metals could be more retained in the protein than when Glycine is used as trailing ion. The estimation of the molecular masses was also correct (16.7 kDa for SOD and 34.5 kDa for ADH) although electrophoresis time is longer than with method 1. The results of LA-ICP-MS scans for SOD and ADH after method 2 are shown in Fig. 2. For this method, the scans were again carried out in duplicate, with the stained and unstained gels. The behaviour of metals in proteins is now quite different. For both proteins, no significant signals of metals (Fig. 2a and c) were obtained after staining the gel. However, observing Fig. 2b, where LA-ICP-MS scan of unstained gel is shown, for SOD, Cu and Zn signals can be appreciated just in the same position of the protein band. This confirms as supported by other authors concerning to the loss of metals when the proteins are exposed to staining and destaining solutions [17,30]. So, in future, it should be avoided to use staining procedures to visualize proteins previously to LA-ICP-MS measurements. One solution could be the same as proposed in this paper that consists of running the electrophoresis in duplicate: one for staining and visualization of proteins and the other one for detecting metals by LA-ICP-MS. The use of Tricine instead of Glycine as trailing ion in denaturing PAGE conditions preserves the metal–protein binding for SOD. This is especially interesting when 2D PAGE is being used, because 2D PAGE protocols rely on the use of denaturing chemicals. As we explain in our previous work [16] at the usual electrophoresis pH values, Tricine migrates much faster than Glycine ($pK_1 = 2.3$ and $pK_2 = 8.15$ for Tricine and dissociation constants for Glycine are $pK_1 = 2.47$ and $pK_2 = 9.66$) and causes less protein movement. On the other hand, complex formation constants for Cu–Glycine and Zn–Glycine are respectively $\log \beta_1$ (Cu) = 8.1, $\log \beta_2$ (Cu) = 15.1 and $\log \beta_1$ (Zn) = 5.0, $\log \beta_2$ (Zn) = 9.2, $\log \beta_3$ (Zn) = 11.6 which are higher than complex formation constants for Cu–Tricine ($\log K = 7.01$) and Zn–Tricine ($\log K = 5.1$) [38]. Consequently Cu–Glycine and Zn–Glycine complexes are stronger than Cu–Tricine and Zn–Tricine complexes. These results confirm that using Glycine as trailing ion the possibility of loosing metal–protein binding is higher than using Tricine as trailing ion.

To see how much Cu and Zn remain bound to SOD after applying SDS–Tris–Tricine-PAGE–LA-ICP-MS, portions of gel corresponding

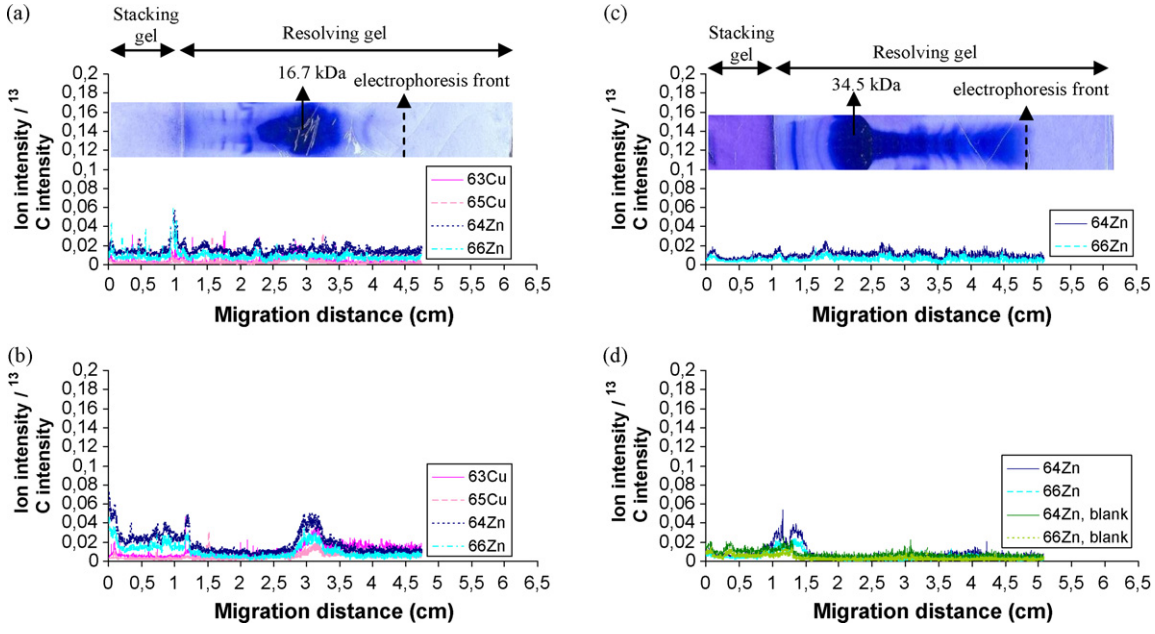

Fig. 2. Detection of metals in proteins by LA-ICP-MS after SDS–Tris–Tricine-PAGE (method 2) (a) Cu and Zn in SOD (stained gel) (b) Cu and Zn in SOD (unstained gels) (c) Zn in ADH (stained gel) and (d) Zn in ADH (unstained gels).

Table 2
Metals concentration determination (mg g^{-1}) in SOD and ADH.

Method	SOD		ADH
	Cu	Zn	Zn
Protein solution by ICP-MS	3.0 ± 0.21	3.7 ± 0.32	3.0 ± 0.23
SDS–Tris–Tricine-PAGE–LA-ICP-MS (30 mA)	0.55 ± 0.05	0.33 ± 0.009	
SDS–Tris–Tricine-PAGE–LA-ICP-MS (20 mA)	2.5 ± 0.33	2.4 ± 0.37	–
AN-Native-PAGE–LA-ICP-MS	2.6 ± 0.27	2.5 ± 0.26	–
BN-Native-PAGE–LA-ICP-MS	2.7 ± 0.25	3.1 ± 0.37	2.6 ± 0.30

to SOD band (8 mg) were digested with a microwave system and Cu and Zn contents were determined. The results are shown in Table 2. Three different gels under same electrophoresis conditions (SDS-Tris-Tricine-PAGE, method 2) were used for quantification. Final concentration was the mean value of the three different gels. The detection limits (based on 3σ criterion of 10 blanks measurements from 10 independent gels) were 0.37 and 2.98 μg g^{-1} for Cu and Zn respectively. Quantification limits were 1.23 and 9.93 μg g^{-1} for Cu and Zn respectively. The found concentration for Cu and Zn in SOD after applying SDS–Tris–Tricine-PAGE–LA-ICP-MS was 2.5 ± 0.33 and 2.4 ± 0.37 mg g^{-1} respectively. In comparison with the total concentration obtained measuring protein solution by ICP-MS (3 ± 0.21 and 3.7 ± 0.32 mg g^{-1} for Cu and Zn) the results are somewhat low. Anyway the concentrations obtained after applying SDS–Tris–Tricine-PAGE–LA-ICP-MS at 20 mA are much higher than the ones obtained at a current of 30 mA with the same gel digestion procedure (0.55 ± 0.05 and 0.33 ± 0.09 mg g^{-1} for Cu and Zn). These results are fairly similar as the ones obtained in our previous work using calibration matrix-matched standards [16] (0.5 ± 0.07 and 0.3 ± 0.1 mg g^{-1} for Cu and Zn). The effect of current applied during electrophoretic process for SOD in unstained gel after SDS–Tris–Tricine-PAGE–LA-ICP-MS using a much higher current (40 mA) is shown in Fig. 3. It can be observed that when using higher current (40 mA) some Zn peak appears just in the same position as SOD protein band, but Cu peak and a small Zn peak appear a little bit moved in relation to protein band. It seems that, at higher intensities, proteins advance faster than lower intensities and even using Tricine as trailing ion, the possibility of metal–protein binding losses are higher. Cu and Zn in SOD band after running SDS–Tris–Tricine-PAGE at a current of 40 mA have been quantified also after gel digestion, being the results obtained 0.37 ± 0.03 and 1.6 ± 0.18 mg g^{-1} for Cu and Zn respectively. These results confirm our predictions in relation to the loss of metal–protein binding under the condition of higher current (40 mA) employed.

No Zn signals in protein band are obtained for ADH after SDS–Tris–Tricine-PAGE–LA-ICP-MS scan of unstained gel either (Fig. 2d). Although some Zn signals appear in the stacking gel (maybe due to some contamination as can be seen in blank signals), none of them corresponds with the protein band. Due to the fact that metal–protein binding in ADH is not strong enough, even using Tricine instead of Glycine as trailing ion with denaturing conditions, the binding is broken. For ADH the use of non-denanturing electrophoresis will be recommended in order to detect Zn by LA-ICP-MS in proteomics studies. Auld et al. [39] pointed out that the structural Zn site of ADH had been assigned a role in the maintenance of the proper tertiary/quaternary structure. Using denaturing conditions, the disintegration of the tetrameric ADH molecule into monomeric units is proportional to the loss of both its enzymatic activity and of its Zn content [40].

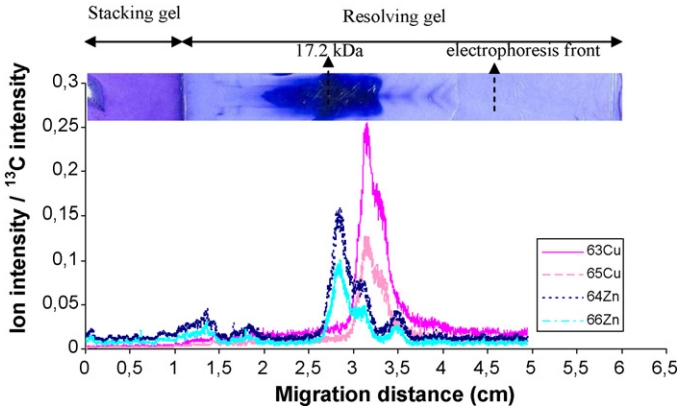

Fig. 3. Detection of metals in SOD by LA-ICP-MS after SDS–Tris–Tricine-PAGE (method 2) (unstained gels) at a current of 40 mA.

3.3. Non-denaturing PAGE conditions

Because of possible metal losses due to the use of denaturing conditions, even when we use Tricine as trailing ion, two different native PAGE methods used before in different works [9,12,14] has also been applied: AN-PAGE (method 3) and BN-PAGE (method 4). They preserve the native protein structure by avoiding the use of denaturing and reductant agents. However, an inconvenience of non-denaturing methods is the no estimation of molecular masses of the proteins because mobilities of the proteins depend now, not only on their molecular masses, but also on their charges. The difference between both methods is fundamentally the trailing ion used. AN-PAGE is based on a Tris–Glycine system and BN-PAGE on a Tris–Tricine system. It is interesting to investigate the behaviour of metal-binding proteins applying both non-denaturing PAGE methods, what has not been reported before in the literature (until our knowledge).

3.3.1. Anodal Native-PAGE (AN-PAGE)

The results obtained by LA-ICP-MS after AN-PAGE were again different in SOD and ADH. The LA-ICP-MS scans for both proteins in unstained gels are shown in Fig. 4. The high background obtained for ^{63}Cu and ^{65}Cu in SOD (Fig. 4a) was due to an uncontrolled contamination in ablation cell. The results showed significative signals for Cu and Zn corresponding to SOD protein band, while no Zn signal was observed in the ADH protein band (Fig. 4b). Again Zn signals can be seen in the zone corresponding to the electrophoresis front. The conclusion we can draw is that using non-denaturing conditions in AN-PAGE based in a Tris/Glycine system, preserves metal–protein binding for SOD (considered a metalloprotein) however, even using non-denaturing conditions (recommended for metal–proteins binding of low affinity) in AN-PAGE where Tris–Glycine is used as trailing ion, Zn is not strongly bound enough to ADH to maintain its integrity during the electrophoretic process.

The content of Cu and Zn bound to SOD after applying AN-PAGE–LA-ICP-MS was also determined digesting portions of gel corresponding to SOD band (10 mg). The results (means of three different gels) are shown in Table 2. The detection limits (based on 3σ criterion of 10 blanks measurements, only one blank gel portion was used) were 0.42 and 5.27 μg g^{-1} for Cu and Zn

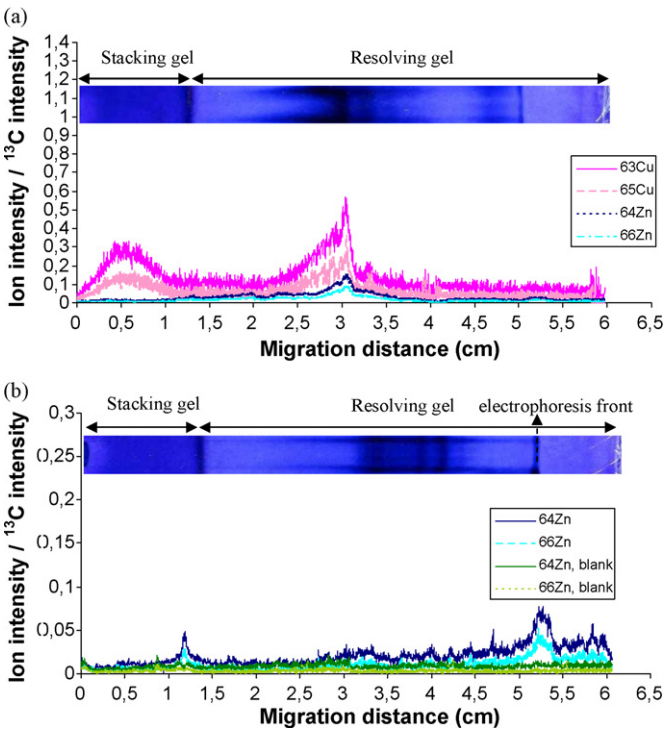

Fig. 4. Detection of metals in proteins by LA-ICP-MS after AN-PAGE (method 3) (unstained gel) (a) Cu and Zn in SOD, and (b) Zn in ADH.

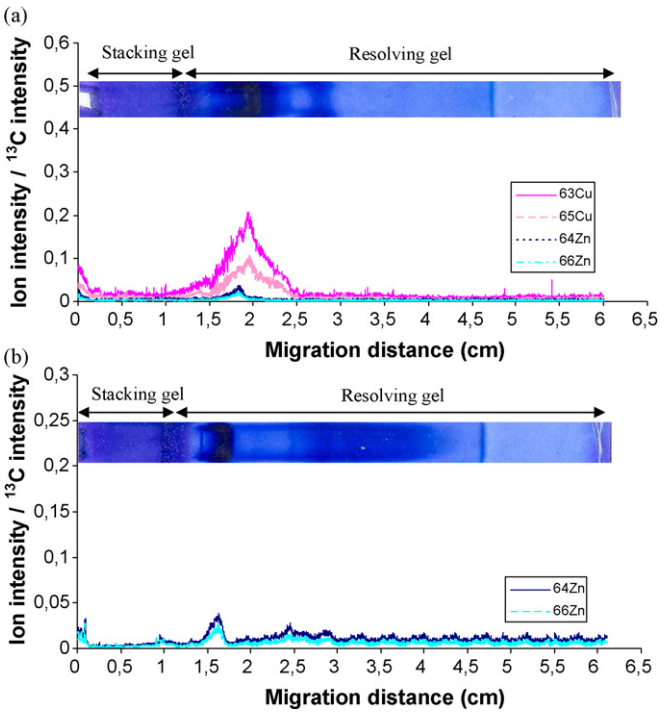

Fig. 5. Detection of metals in proteins by LA-ICP-MS after BN-PAGE (method 4) (unstained gels) (a) Cu and Zn in SOD, and (b) Zn in ADH.

respectively. These values are higher than the ones obtained for blank gels in SDS–Tris–Tricine system (0.31 and 2.92 $\mu g\,g^{-1}$ for Cu and Zn respectively). In AN-PAGE samples were mixed (1:1 v/v) with 600 mM Tris buffer (pH 8.9), and in Tris–Tricine system proteins were denatured in 50 mM Tris (pH 6.8) buffer containing 4% SDS and 2% 2-mercaptoetanol at 100 °C for 5 min. It was observed that as higher the concentration of Tris buffer higher is the metal contamination, especially for Zn. The concentrations obtained for Cu and Zn in SOD after applying AN-PAGE–LA-ICP-MS was 2.6 ± 0.27 and $2.5 \pm 0.26\,mg\,g^{-1}$ respectively, very similar to the ones obtained in denaturing conditions using a Tris–Tricine system. The conclusion is that for SOD you can use either denaturing (but using Tris–Tricine as trailing ion) or non-denaturing electrophoresis conditions followed by LA-ICP-MS for detecting metals in proteomics studies.

3.3.2. *Blue Native-PAGE (BN-PAGE)*

The results of BN-PAGE (based in a Tris/Tricine system) are shown in Fig. 5 (unstained gels). For both SOD (Fig. 5a) and ADH (Fig. 5b) significant signals of Cu and Zn in protein bands were found. Again the high background obtained for ^{63}Cu and ^{65}Cu in SOD was due to an uncontrolled contamination in ablation cell. As we said before, one of the main differences between AN-PAGE and BN-PAGE is the trailing ion used (in BN-PAGE, Tricine is used as trailing ion). For preserving metal–protein binding in ADH not only non-denaturing conditions are necessary but also the use of Tricine as trailing ion. Even with these conditions some Zn signal appear out of the protein band. Applying BN-PAGE–LA-ICP-MS, the content of Cu and Zn for SOD and Zn for ADH corresponding to the protein bands was also determined (see Table 2). The detection limits (based on 3σ criterion of 10 blanks measurements, only one blank gel portion was used) were 0.35 and 3.15 $\mu g\,g^{-1}$ for Cu and Zn respectively very similar as the ones obtained for blank gels in SDS–Tris–Tricine system (same concentration of Tris buffer was used). The concentrations obtained for Cu and Zn in SOD after applying BN-PAGE–LA-ICP-MS were 2.7 ± 0.25 and $3.1 \pm 0.37\,mg\,g^{-1}$ respectively, very close values to the ones obtained for proteins solutions by ICP-MS (3 ± 0.21 and $3.7 \pm 0.32\,mg\,g^{-1}$). The concentration obtained for Zn in ADH was $2.6 \pm 30\,mg\,g^{-1}$ also very close to the one obtained for ADH solution by ICP-MS ($3 \pm 0.23\,mg\,g^{-1}$). This means that most of the Zn is bound to the protein after applying BN-PAGE–LA-ICP-MS based in a Tris–Tricine system.

4. Conclusions

The coupling of gel electrophoresis to LA-ICP-MS can be a very useful technique to determine metals bound to proteins of biological and environmental interest. However, a great pitfall using gel electrophoresis is metal losses during this process and the subsequent impossibility to detect them bound to the proteins.

We have studied some aspects of the separation process in an attempt to find the suitable electrophoretic procedure for two proteins with different metal–protein affinities (superoxide dismutase and alcohol dehydrogenase) to maintain the integrity of the metal–protein complex and detect successfully the metal bound to each protein. We have focused not only on the nature of the electrophoretic process and the staining and destaining procedures, but also on the trailing ion chosen and current applied in the electrophoretic method. Different electrophoretic techniques both denaturing and non-denaturing were applied. In the denaturing ones, Tricine seems to be a better option as trailing ion due to the fact that it seems to have less affinity for Cu and Zn than Glycine and preserves better metal–protein binding due to a slower movement of protein. With respect to current used, it has been observed that as higher current is applied the possibility of metal–protein binding losses is higher. Two non-denaturing methods using different trailing ions (AN-PAGE, and BN-PAGE) were applied and the behaviour of both proteins (SOD and ADH) was studied. Non-denaturing conditions (based on the use of a Tris/Tricine system) were demonstrated to be more suitable for the later determination by LA-ICP-MS of metals such as Zn non-covalently complexed to proteins. Furthermore, the results showed that it is better to avoid staining procedures because they can alter the stability of the

metal–protein complexes and prevent detection of metals bound to proteins.

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