Sensitive Detection of Selenoproteins in Gel Electrophoresis by High Repetition Rate Femtosecond Laser Ablation−Inductively Coupled Plasma Mass Spectrometry

ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2007

Impact Factor: 5.64 · DOI: 10.1021/ac0709145 · Source: PubMed

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Multimode detection (LA-ICP-MS, MALDI-MS and nanoHPLC-ESI-MS^2) in 1D and 2D gel electrophoresis for selenium-containing proteins

Guillaume Ballihaut, Christophe Pécheyran, Sandra Mounicou, Hugues Preud'homme, Régis Grimaud, Ryszard Lobinski

Speciation of heteroelement-containing proteins essential for human health is one of the most rapidly developing areas in bio-inorganic analytical chemistry (metallomics). We discuss recent advances in mass spectrometry (MS) techniques for detection and identification of selenium (Se)-containing proteins separated by gel electrophoresis (GE). These advances include location of Se-containing bands in 1D GE or spots in 2D GE by laser-ablation inductively coupled plasma MS (LA-ICP-MS) followed by protein identification by matrix-assisted laser-desorption/ionization MS (MALDI-MS) and nano-high-performance liquid chromatography-electrospray-tandem MS (nanoHPLC-ESI-MS^2). We highlight the differences with the classical proteomics approach resulting from the presence of the Se atom.

Keywords: Gel electrophoresis; Inductively-coupled plasma; Laser ablation; Mass spectrometry; Multimode detection; Nano high-performance liquid chromatography; Protein; Selenium; Tandem mass spectrometry

Guillaume Ballihaut*, Christophe Pécheyran, Sandra Mounicou, Hugues Preud'homme, Ryszard Lobinski*
Laboratoire de Chimie Analytique Bio-inorganique et Environnement (UMR 5034)
Hélioparc, 2, av. Pr. Angot, F-64053 France

Guillaume Ballihaut, Régis Grimaud
Laboratoire d'Ecologie Moléculaire (Microbiologie),
av. de l'Université,
B.P. 1155, 64013 Pau, France

Ryszard Lobinski
Department of Analytical Chemistry,
Warsaw University of Technology,
ul. Noakowskiego 3, 00-664 Warszawa, Poland

*Corresponding authors. Tel.: +33 5 59 80 68 84; Fax: +33 5 59 40 77 81;
E-mail: guillaumeballihaut@yahoo.fr (G. Ballihaut), Ryszard.Lobinski@univ-pau.fr (R. Lobinski).

1. Introduction

Speciation of protein-bound elements is one of the most rapidly developing areas in bio-inorganic analytical chemistry (metallomics) [1–3]. Slab-gel electrophoresis (GE) based on isoelectric focusing or simple migration in the gel (Native and SDS PAGE), in one or two dimensional format, has been the principal separation technique for high-resolution protein separation [4,5]. Heteroelements have been specifically detected in the gels by autoradiography of radioactive (e.g., ^{75}Se-labelled) species [6,7] but atomic spectrometric techniques, especially laser-ablation inductively coupled plasma MS (LA-ICP-MS) detection, have become established options [8].

The presence of selenium (Se) in proteins may lead to selenoproteins, which contain the genetically encoded amino-acid selenocysteine (ex. glutathione peroxidase, thioredoxine reductase, selenoproteins P and W) and to other Se-containing proteins, in which selenomethionine and selenocysteine non-specifically replace methionine and cysteine, respectively. Selenoproteins usually contain a single SeCys residue (Selenoprotein P is a notable exception here). In the case of non-specific incorporation, an Se-containing protein is usually accompanied by a considerable excess of its methionine or cysteine analogue.

Selenoproteins exist naturally in all kingdoms of life and are of particular

0165-9936/$ - see front matter doi:10.1016/j.trac.2007.01.007

interest because of the recognized essentiality of Se in human nutrition and the putative cancer-prevention properties [9–11]. Understanding the reactions and the functions of selenoproteins is considered a possible key to the mechanisms of essentiality and toxicity of Se.

The spectacular progress in DNA sequencing has resulted in the availability of whole-genome sequences of an increasing number of organisms. Bioinformatic analysis of these sequences has allowed the prediction of many new selenoproteins [12,13]. The development of methods allowing their detection and quantification *in vivo* is becoming of paramount importance.

Below is an overview of current trends in analytical methodology concerning the detection and identification of Se-containing proteins in gel electrophoresis based on recent data from our laboratory.

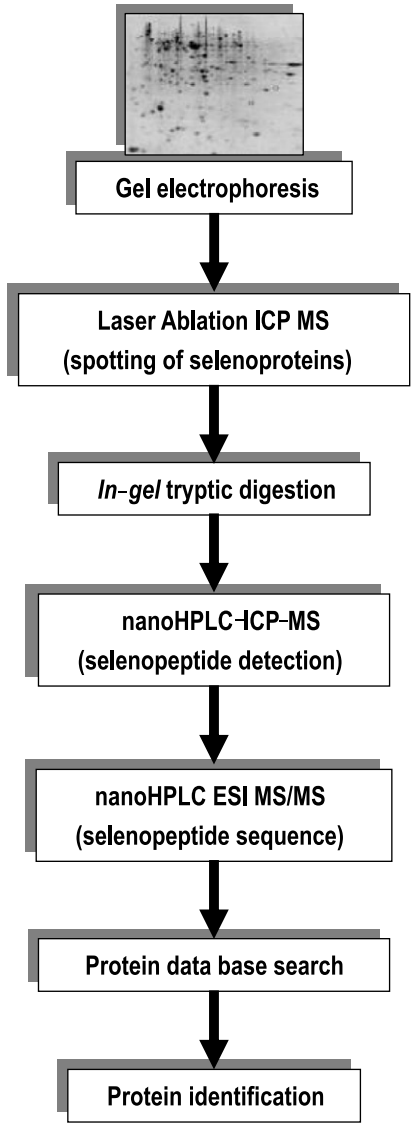

Figure 1. Analytical workflows in selenoproteomics.

2. State-of the-art of gel-electrophoresis-based analytical techniques for selenoproteomics

The principle of the approach (Fig. 1) is based on a classical workflow in proteomics in which the proteins separated by 1D or 2D gel electrophoresis are subject to in-gel digestion with trypsin followed by matrix-assisted laser-desorption/ionization (MALDI) (if the sequenced genome of the organism is known) and/or nano-high-performance liquid chromatography-electrospray-tandem MS (nanoHPLC-ESI-MS^2) analysis (also for *de novo* sequencing). As Se is covalently bound, the limitations of the technique are the same as classical proteomics. Additional problems are due to selenomethionine and selenocysteine being more prone to oxidation than their sulphur analogues, and the selenol residues undergo elimination more easily than the thiol residues [14].

Applying a classical proteomics approach to selenoproteomics usually fails as very few of the 1000 proteins contained within the gel actually contain Se, and it is these few that are of interest. In addition, the concentration of selenoproteins in the cell or in the extract is not usually higher than the upper picomolar range, so they are often accompanied in the gel by the non-selenized, more abundant proteins that are responsible for the appearance of the band or spot. Consequently, unless the protein is suitably enriched and purified, the ionization of Se peptides produced by its tryptic digestion is likely to be suppressed by the peptides from concomitant proteins present in excess in the band or even in the spot.

As has been shown in Fig. 1, the stated problems can be addressed at two levels:

(i) the specific detection of Se-proteins in the gel by LA-ICP-MS in order to pick up targets for further characterization; and,
(ii) the specific detection of Se-containing peptides in tryptic digests of the proteins from the gel to focus the sequence analysis on Se-containing peptides.

LA-ICP-MS was first applied in the context of selenoproteome research to reveal a number of Se-containing proteins in a waterfowl embryo and fish ovary collected from contaminated sites [15]. The method was later applied to the detection of glutathione peroxidase isolated from red blood cells [16], and extended to 2D gels of selenized-yeast proteins [16]. A comprehensive study by Chassaigne et al. showed 2D-GE-LA-ICP-MS mapping of Se-containing proteins in a selenized yeast extract [17]. The advances in capillary HPLC [18,19] allowed specific peptide mapping by capillary HPLC-ICP-MS from the tryptic digests of proteins extracted from gel bands [18] or spots [19]. The peptides were identified by matching

the retention time of peaks in the HPLC-ICP-MS chromatogram of the in-gel digest with those present in the reference chromatogram of the purified and characterized protein [19]. The principal problems concern:

(i) quantification because well-characterized standards of Se-proteins are unavailable; and,
(ii) often insufficient sensitivity (most of developments were shown for the selenized yeast in which the concentration of protein-bound Se exceeded 1000 μg/g).

An even bigger challenge, that has so far been addressed with only limited success, is identification of the proteins extracted from the gel. Studies making it possible to distinguish between selenomethionine and selenocysteine in protein bands are scarce [20]. ESI mass spectra of intact proteins have been collected from the spots in which Se had been detected [17]. However, it is ambiguous as to whether they correspond to Se-containing proteins or to other proteins present in the spot in which Se was detected.

Spectra of peptides from purified proteins have been obtained from MALDI-MS [14,21,22] and ESI-TOF-MS [22,23]. However, a number of problems seem to prevent successful application of these techniques on proteins extracted from gels. They include an insufficient amount of extracted protein and suppression of ionization. Hence, there is interest in parallel ICP-MS detection that provides information on whether Se-containing peptides are present, how many and how much, and gives the retention time for the peptides to be identified by TOF-MS.

3. Protein detection and identification

3.1. LA-ICP-MS

All the applications to date used UV lasers (193 nm ArF, 266 nm Nd:YAG) at a pulse width in the low-ns range and repetition rate of 5–20 Hz. In our experiments, scans at a speed of 50–100 μm/s resulted in an ablation zone (crater diameter) of ca. 100–150 μm and the depth of ca. 500 μm.

Fig. 2 demonstrates that ablation at these conditions is a linear function of concentration. Quantification can be carried out using external calibration without the need for more sophisticated techniques. The use of an ICP mass spectrometer equipped with a collision cell is essential to allow interference-free detection of the most-abundant ^{80}Se isotope, which, in turn, is the prerequisite of attaining the sub-pg (as Se) detection limits. The high sensitivity of LA-ICP-MS detection allows detection of proteins that cannot be detected by Coomassie Blue staining.

The sensitivity of Se detection can be increased by increasing the amount of protein that is ablated and transported into the ICP. It can be achieved (e.g., by

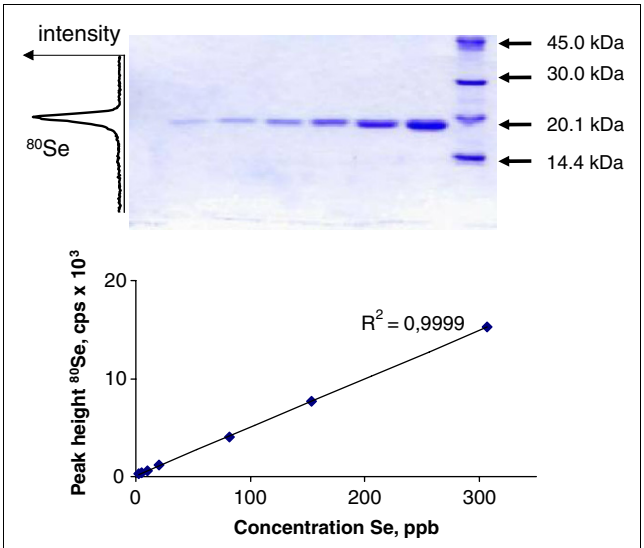

Figure 2. Nanosecond LA-ICP-MS selenium detection in 1D SDS PAGE. The calibration curve is a linear function of the quantity of protein introduced on the gel.

using an fs infra-red laser (λ = 1030 nm) working at a much higher repetition rate (10 kHz) (Novalase, Sa, France) [24]. The energy per pulse is much lower (31 μJ instead of 1.6 mJ), and the fluence is maintained at high level (13 J/cm^2) due to a small spot size (<20 μm) for a better ablation yield. The combination of the high repetition rate and a fast scanning-beam device (patent pending) allows ablating quasi-instantaneously (ICP-MS detection timescale) in relatively large zones. The LA lane width can then reach 2 mm instead of <200 μm with the standard ns laser. The particle size is typically smaller, which facilitates their transport and atomization in the plasma. Fig. 3a compares the sensitivity of ns and fs LA for detecting a selenoprotein, formate dehydrogenase, which contains a single Se atom per 1 molecule (ca. 79 kDa) of the protein. For the GE ns LA-ICP-MS coupling, detection limits were in the sub-pg range [18]. This value is comparable in absolute terms with that attainable by using fs LA-ICP-MS when the same ICP-MS detector is used. However, as distinctly more matter can be ablated in a time unit by fs LA-ICP-MS, the signal-to-background ratio is considerably higher (ca. 20 times) than in the ns LA-ICP-MS.

Detection by fs LA-ICP-MS allows sensitive and fine studies of the incorporation of Se into proteins. The example shown in Fig. 3b shows the different incorporation patterns in *E. coli* grown in medium containing selenomethionine and selenite, the left and right panels, respectively. Note that an overlap between the protein pattern and the Se-distribution pattern does not always occur. Se may be incorporated into minor proteins that are not detectable by the Coomassie Blue staining.

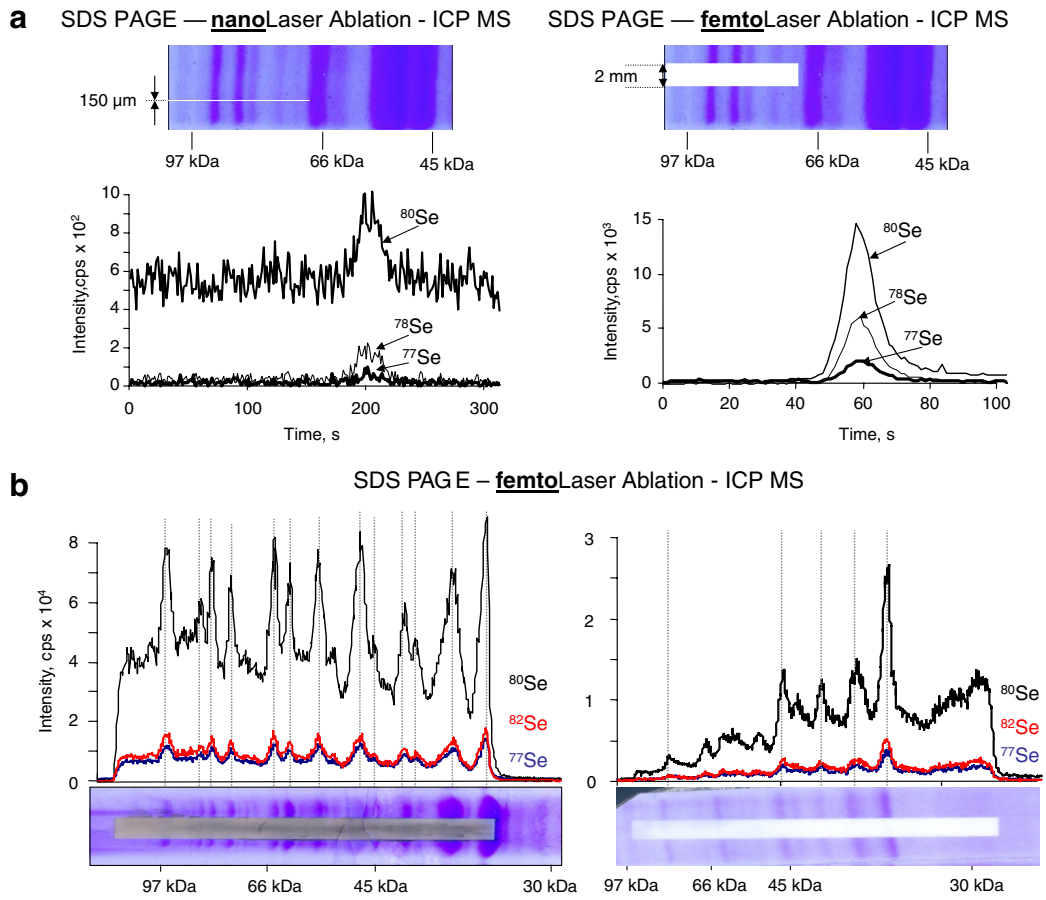

Figure 3. Fs LA-ICP-MS in selenoproteomics. (a) Comparison of the sensitivity using ns and fs lasers; SDS PAGE analysis of a size-exclusion fraction containing formate dehydrogenase expressed in *Escherichia coli*. (b) Comparison of Se-incorporation in proteins during *E. coli* growth in culture media containing selenite and selenomethionine.

3.2. MALDI-TOF-MS

Efficient extraction of intact proteins from the gel is difficult. Se-containing protein extraction was claimed by Chassaigne et al. [17] but neither the recovery or the presence of Se was seen in the ESI mass spectra. The problem is that a Se-containing protein can be accompanied at least by its methionine analogue, and the latter is more abundant and ionized preferentially.

The use of trypsin to digest the original protein in gel is a well-known procedure for efficient protein extraction prior to molecular MS analysis [25]. As Se is covalently bound, it is present in the fragments issued from the tryptic digestion. Carboxymethylation is essential to protect the selenol groups in SeCys-containing selenoproteins but the derivatization agent may react with selenomethionine [26] and produce artefact peaks.

Fig. 4 shows a MALDI-MS spectrum obtained after extraction of the Se-calmoduline spot and the zooms on Se-containing peptides. As can be seen from the full-range mass spectrum (Fig. 4a), the Se compounds are not the major ones, and isotopic patterns characteristic to selenocompounds cannot be recognized. Only careful selection of appropriate mass ranges (requiring prior knowledge of the protein sequence) allows detection of the Se-containing compounds in the extract. The sequence coverage is almost complete (except for T4 and T7 peptides) in terms of Se-containing peptides. Taking into account the amount of the protein present in the spot (1.34 µg), the detection limits are poor. They are negatively affected by:

- ionization of SeMet-containing peptides that is apparently poorer than that of their sulphur analogues;
- the rich isotopic pattern of Se; and,
- the multi-isotopic peaks present.

Note that no success was obtained for detection by MALDI-MS of the peptides extracted from the same amount of protein separated by 1D sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (without isoelectric focusing (IEF)) because some compounds extracted from the gels were apparently sufficient to suppress the signal.

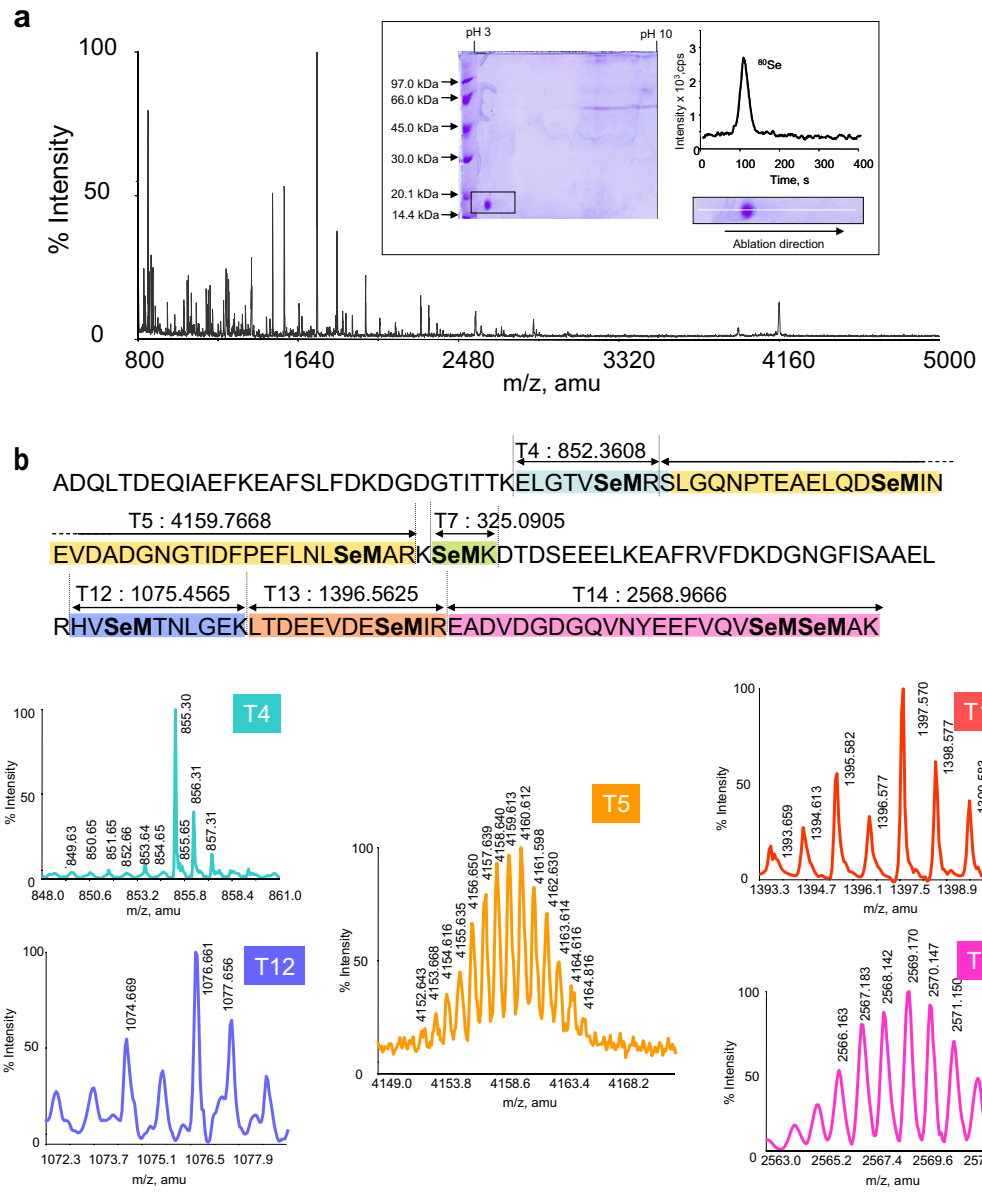

Figure 4. MALDI-TOF-MS mapping of Se-containing peptides after in-gel tryptic digestion of selenomethionyl calmodulin isolated by 2D gel electrophoresis. (a) Broad range mass spectrum; and, (b) protein sequence and zooms of mass spectra corresponding to the Se-containing fragments present. The inset shows the image of the gel, the ablation trace through the spot, and the analytical signal.

3.3. NanoHPLC with parallel ICP-MS and ESI-MS

Se peptides in the tryptic digest can be identified by nanoHPLC-ESI-MS^2. Our experience shows that the success of this approach strongly depends on the purity of the sample analyzed. In order to be detected, a minor selenopeptide must arrive virtually pure at the source at a given moment. Selenocompounds are located in the mass spectrum because of their isotopic patterns [27]. The search is manual so knowledge of the retention time is extremely helpful unless the protein is known and a particular ion can be searched for in order to generate an extracted ion chromatogram. The retention time at which an Se compound should be detected can be determined by parallel ICP-MS detection [27,28]. An unbeatable advantage of ICP-MS detection is sub-femtomolar sensitivity, regardless of the compound structure, quantitative response, and the lack of ionization suppression, even in the presence of a high concentration of co-eluting compounds. Hence, ICP-MS detection gives information about the number of compounds that should be identified by ESI-MS^2, their concentrations, the digestion efficiency, and the recovery from the column.

Fig. 5 illustrates principle of 1D-GE-LA-ICP-MS followed by nanoHPLC with parallel ICP-MS and ESI-MS^2 detection

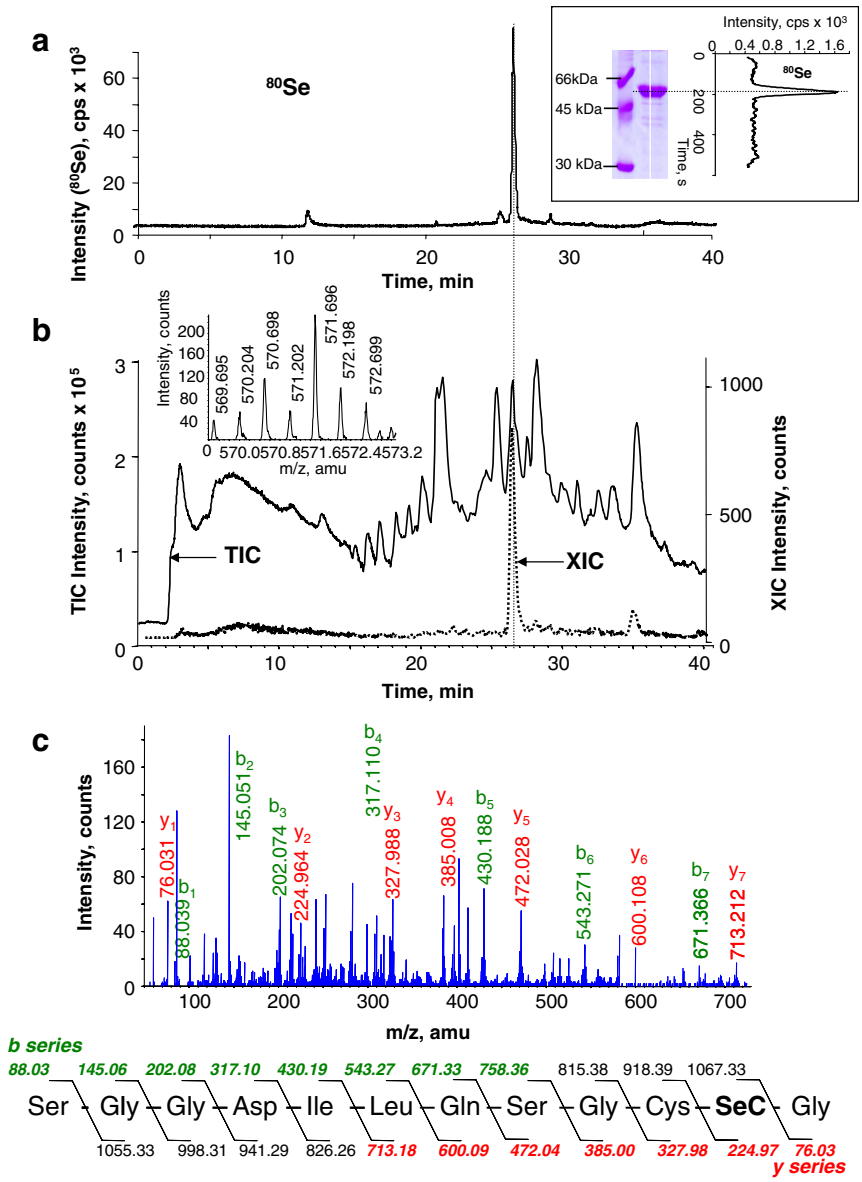

Figure 5. Identification of thioredoxine reductase separated by gel electrophoresis by nanoLC after on-line preconcentration with the parallel ICP-MS and ESI-MS^2 detection. (a) NanoHPLC-ICP-MS chromatogram (the gel and its LA-ICP-MS image are shown in the inset). (b) nano-HPLC-ESI-MS chromatogram. Solid line: TIC; dotted line: XIC. The Se-isotopic pattern of the double-charged ion corresponding to the XIC is shown in the inset. (c) CID mass spectrum and the sequence of the selenopeptide.

for identification of rat thioredoxine reductase in the gel. The protein detected in the band by ns LA-ICP-MS (Fig. 5, inset in the top panel) was recovered by enzymatic digestion and analyzed by nanoHPLC-ICP-MS using a set-up described elsewhere [28]. It produced a single intense peak corresponding to the selenopeptide peptide (only one was expected on the basis of the protein-database sequence). The middle panel shows a total ion chromatogram (TIC): the signal-to-noise ratio in the extracted ion chromatogram (XIC) of the ion corresponding to the peptide (see inset) is comparable to that with ICP-MS detection. This is because of the high purity of the Se-protein standard used. The thioredoxine reductase did not need to be derivatized because of the presence of an internal S-Se bridge. The bottom panel of Fig. 5 confirms the identity of the peptide by MS^2. Most of *b*-series (green) and *y*-series (red) ions could be detected. This approach is sensitive (pg level) and can be considered as generic on the condition that the protein is well purified.

Another example of this approach shown in Fig. 6 concerns selenomethionyl calmodulin extracted from the 2D electrophoretic gel. NanoHPLC-ICP-MS shows four peaks out of six that should be present (*cf.* the sequence in Fig. 4). The smallest SeMetK dipeptide was not hydrophobic enough to be trapped on the preconcentration column. The biggest, T5, could not be

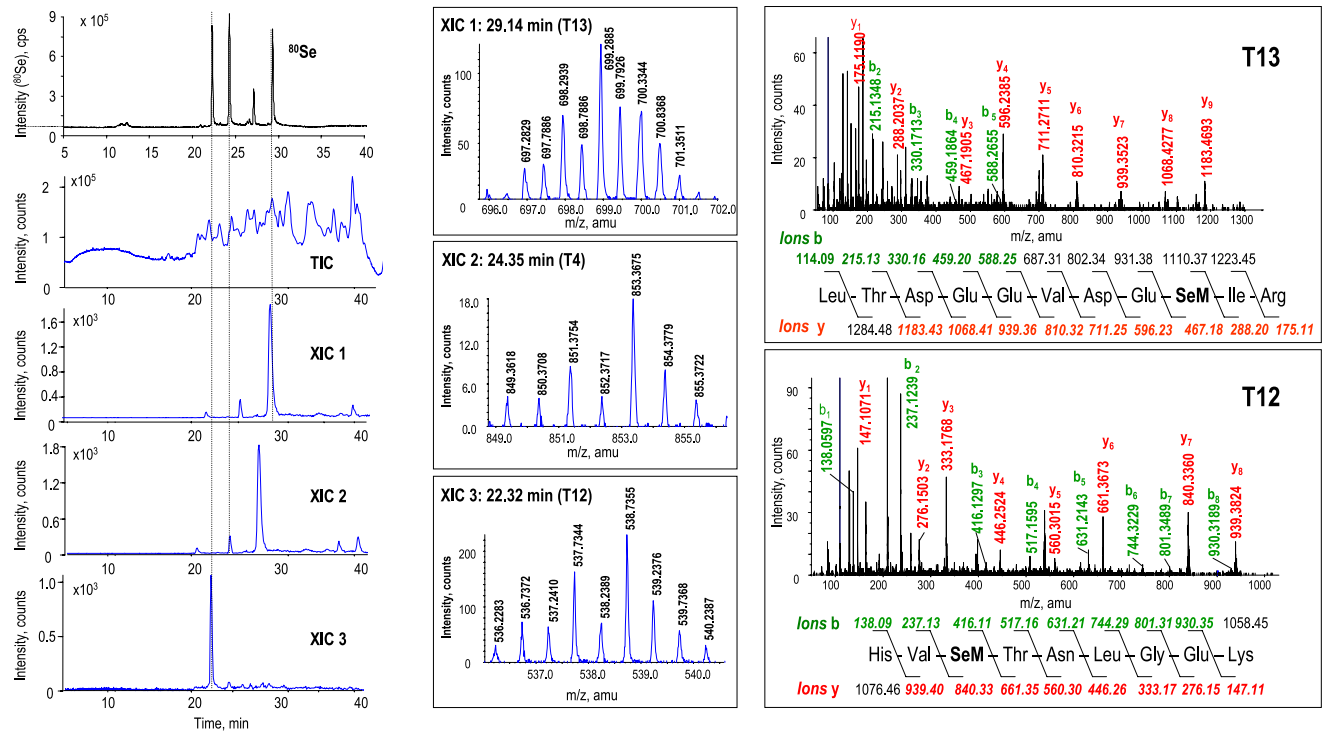

Figure 6. Identification of selenomethionyl calmoduline separated by 2D gel electrophoresis by nanoLC after on-line preconcentration with parallel ICP-MS and ESI-MS^2 detection (cf. inset to Fig. 4 for the gel image). (a) 1st panel from the top: nanoHPLC-ICP-MS chromatogram; 2nd panel from the top: nanoHPLC-ESI-MS chromatogram; 3,4 and 5th panels: extracted ion chromatograms at m/z 699.3 (XIC 1), 853.4 (XIC 2), and 538.74 (XIC 3), respectively. (b) Mass spectra at the apexes of the extracted ion chromatograms (XIC 1 – XIC 3); (c) CID mass spectra of the double-charged ions m/z 699.3 and m/z 538.74 and the corresponding sequences.

detected for unknown reason. Of the four peaks detected, only three could be detected by ESI-MS. No Se pattern was identified in peak 3 probably because it was insufficiently pure. Only the signal-to-noise ratio of peptides T12 and T13 was enough to obtain sufficiently intense MS^2 spectra and to determine the peptide sequences. The intensity of the T4 peptide did not allow a reliable MS^2 spectrum to be obtained. This example shows the importance of the nanoHPLC-ICP-MS step to double check whether the lack of a signal in ESI-MS^2 detection is due to the absence of the peptide or the suppression of ionization by the concomitant species.

4. Conclusions

The recent advances in analytical methodology seem to be crucial in accelerating progress in the specific detection and identification of selenoproteins *in vivo*. Fs LA-ICP-MS appears to be the most sensitive detection technique for GE because of the capability of introducing considerably more material in a given time than when using ns LA. In contrast to radioactive-tracer-detection methods, GE-LA-ICP-MS is applicable to human samples. NanoHPLC-ICP-MS analysis is a valuable method for the quantitative determination of the recovered peptides from bands or spots and very helpful for targeting peptides for ESI-MS^2 analysis. The sensitivity of MALDI-MS is sufficient for tryptic peptides originated from only well-purified and relatively concentrated proteins.

Acknowledgements

This work was financially supported by ANR (Agence Nationale de la Recherche). Support of the Aquitaine Region for the MS platform is acknowledged. Guillaume Ballihaut acknowledges the Ph.D. fellowship from the French Ministry of Research. The authors thank Dr. Elias Arner (Karolinska Institute, Stockholm, Sweden) for the gift of thioredoxine reductase and Judith Richter (LEM, UPPA) for technical help with gel electrophoresis and *E. coli* culture.

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Guillaume Ballihaut obtained his M.Sc. degree from the University of Pau, France, in 2003, and is currently finishing his Ph.D. at the same institution. His research is focused on the detection of selenoproteins in bacteria.

Christophe Pécheyran is research engineer at the CNRS (French National Research Council) working on the development of fs LA for sample introduction into the ICP.

Sandra Mounicou is CNRS research scientist working in the area of Se- and metal-protein complexes in biological systems.

Hugues Preud'homme is engineer at the CNRS, in charge of the MS platform at the UMR 5034 in Pau.

Régis Grimaud is lecturer in the Department of Biology at the University of Pau.

Ryszard Lobinski is research director at the CNRS and full professor at the Warsaw University of Technology. He leads the Group of Bioinorganic Analytical Chemistry at the UMR 5034 in Pau dealing with topics related to element speciation in biological systems, metalloproteomics and metallomics.